KENMERKEN, VERSPREIDING EN VOORTBESTAAN VAN RANA LESSONAE CAMERANO, RANA RIDIBUNDA PALLAS EN HUN HYBRIDE "RANA ESCULENTA" LINNAEUS (AMPHIBIA, ANURA) IN NEDERLAND

H.E.J. WIJNANDS

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PROMOTOR:

DR. H.C.J. OOMEN

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P R O E F S C H R I F T

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. P.G.A.B. WIJDEVELD, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 1 FEBRUARI 1979 DES NAMIDDAGS TE 4 UUR

door

HENDRIK ELISABETH JOSEPH WIJNANDS
geboren te Heerlen

1979

Druk: Offsetdrukkerij Faculteit der Wiskunde en Natuurwetenschappen
Nijmegen

DANK,

aan allen die mij bij de voorbereiding van mijn proefschrift hebben geholpen, vooral de afdeling Dieroecologie, waarvan ik speciaal Els Derksen wil noemen, die verreweg het grootste gedeelte van het manuscript heeft getypt,
verder de studenten die in het kader van hun doctoraalstudie of tijdens excursies aan het onderzoek hebben meegewerkt: Johan Bekhuis, Harrie van Betuw, Rosemarie van den Brand, Martin van der Burgt, Ton Debets, Wim van Dijk, Pierre Evers, Peter Gerrits, Sjef Goossens, Guido van Geel, Rini de Goey, Koen Govers, Henk Husslage, Alex Janssen, Peter Kleuters, Johan Las, Gerard Mangnus, Trudy Menting, Joop van den Oord, Hay Peeters, Theo Platel, Jean Schellinx,
en tenslotte alle personen en instanties wier toestemming nodig was om kikkers te vangen.

Aan mijn ouders

INHOUD

INLEIDING

Onderzoek van de laatste vijftien jaar heeft ertoe geleid dat *Rana esculenta*, de groene kikker, niet langer als soort wordt beschouwd maar als hybride van *Rana lessonae*, die wel kleine groene kikker genoemd wordt, en *Rana ridibunda*, de meerkikker. Dit is op zichzelf nog niet zo'n interessante constatering. Het is immers niet ongewoon dat de status van een taxon gewijzigd wordt op grond van nieuwe gegevens, terwijl kruisingen tussen verschillende soorten wel vaker voorkomen in de natuur. Natuurlijke interspecifieke kruisingen leveren echter, volgens een gangbare definitie, geen vruchtbare nakomelingen op en dat is wel zo, zij het niet onvoorwaardelijk, in het geval van het *Rana esculenta* complex, zoals de groep in kwestie tegenwoordig wordt genoemd. Dit zou een reden kunnen zijn om het complex als één soort te beschouwen. Er zijn daarentegen meer argumenten, op morfologisch, serologisch, enzymologisch, karyologisch, oecologisch en genetisch vlak, die ervoor pleiten dat *Rana esculenta* oorspronkelijk ontstaan is door hybridisatie van *Rana lessonae* en *Rana ridibunda* (GÜNTHER 1970; BERGER 1973; TUNNER 1973; UZZELL & BERGER 1975). De laatste twee kunnen volgens GÜNTHER (1973) het beste als semispecies worden beschouwd.

Gemeten aan de grootte van het verspreidingsgebied - vrijwel geheel West-, Midden- en Oost-Europa (HOTZ 1974) - en het talrijke voorkomen is *Rana esculenta* een zeer succesvolle vorm: met het oog op zijn hybride-status een interessant gegeven. Het is dan ook niet verwonderlijk dat op verscheidene plaatsen in Europa onderzoek wordt gedaan naar zijn ontstaan en ontwikkeling, waarbij vooral aandacht wordt besteed aan genetische aspecten. De resultaten van deze onderzoekingen wijzen erop dat *Rana esculenta* niet overal op dezelfde wijze in stand wordt gehouden. Volgens TUNNER (1974) treedt bij *Rana esculenta* (in Oostenrijk) hybridogenese op. Dit is een proces waarbij het genoom van één der ouders tijdens de gametogenese (van de hybride) in zijn geheel wordt uitgestoten (SCHULTZ 1969). Terugkruising van de hybride met die oudersoort zorgt dan voor de produktie van nieuwe hybriden. GÜNTHER (1975 a en b) concludeert echter op grond van zijn bevindingen (in Oost-Duitsland) dat bij *Rana esculenta* ook recombinatie en selectieve processen op kunnen treden. Bovendien vindt hij *esculenta*-populaties die kennelijk in staat zijn zichzèlf in stand te houden. Kruisingen tussen *lessonae* en *ridibunda* spelen tegenwoordig waarschijnlijk nergens een rol van betekenis voor de produktie van *esculenta*.

In Nederland is nog nauwelijks aandacht geschonken aan deze problematiek. Dit proefschrift levert hieraan een bijdrage en wil vooral het voortbestaan van de hybride *esculenta*-vorm vanuit oecologisch standpunt belichten.

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BIOMETRICAL AND SEROLOGICAL EVIDENCE FOR THE OCCURRENCE OF THREE PHENOTYPES OF GREEN FROGS (RANA ESCULENTA COMPLEX) IN THE NETHERLANDS

by

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SUMMARY

Serological and biometrical properties of 183 green frogs from two different localities in the Netherlands were examined, in order to assess whether they belong to one or more of the three forms of *Rana esculenta* complex *(lessonae, esculenta* and *ridibunda)*.

Three albumin patterns were found which, using discriminant analysis, could be predicted with sufficient reliability on the basis of a few biometrical measures.

It is stated that a frog can only be identified on the basis of agreement between its biometrical and serological characterization. In this way 160 frogs could be identified. It is concluded that *lessonae, esculenta* and *ridibunda* occur in the Netherlands.

INTRODUCTION

In Europe three forms of green frogs can be distinguished: the forms *esculenta, ridibunda* and *lessonae*. Originally they have been described as *Rana esculenta* Linnaeus 1758, *Rana ridibunda* Pallas 1771 and *Rana esculenta* var. *lessonae* Camerano 1882 respectively. However, throughout the years there has been little agreement on their taxonomic status. They have been regarded as belonging to one species, *Rana esculenta* by BOULENGER (1891, 1918) and KAURI (1954, 1959); to two species, *Rana ridibunda* and *Rana esculenta* by MERTENS & WERMUTH (1960) and GÜNTHER (1968); or to three species, *Rana ridibunda, Rana esculenta* and *Rana lessonae* by KARAMAN (1948) and BERGER & MICHALOWSKI (1971). The most recent hypothesis has come from BERGER (1968, 1970, 1971a, 1971b, 1973) who, on the basis of results of experimental crosses between the three forms, states that: "...*Rana lessonae* and *Rana ridibunda* are taxa at species rank, whereas *Rana esculenta* is a hybrid resulting from interspecific crosses between *Rana lessonae* and *Rana ridibunda*" (BERGER, 1973). This hypothesis is supported by the fact that GÜNTHER (1970) discovered a high number of triploids within the *esculenta* form and by the results of a study of plasma albumins in the three forms referred to above (TUNNER, 1970, 1972 and 1973).

Green frogs in the Netherlands have mostly been called *Rana esculenta* and little attention has, thus far, been paid to the possible occurrence within this form of diverse types. The only reliable notion on the occurrence of green frogs other than *Rana esculenta* was published by HOOGMOED (1975), who described two specimens of *Rana ridibunda ridibunda* from Surhuisterveen (Province of Friesland) and Leiden (Province of Zuid-Holland). This lack of knowledge prompted us to undertake an investigation concerning the identity of green frogs in the Netherlands. This study is, furthermore, part of a series of ecological investigations by our laboratory, focussing mainly on populations of amphibians in the "Overasseltse en Hatertse vennen" (VAN GELDER & HOEDEMAEKERS, 1971; VAN GELDER, 1973).

It was decided to collect biometrical information of the frogs and compare these with serological data obtained from the same individuals.

MATERIAL AND METHODS

Collecting stations

Frogs were caught in two different parts of the country. One area is the State Reserve "Overasseltse en Hatertse vennen", situated at 5°48′ E.Long. 51°48′ N.Lat. in the Province of Gelderland. It consists of afforested sanddunes with oligotrophic and mesotrophic fens. Mainly in the latter fens 118 frogs were caught during the breeding season of 1974. The other area concerns the Lake District in the Province of Friesland between 5°30′ and 5°52′ E.Long. and 52°53′ and 53° N.Lat. Here 65 frogs were caught mainly in ditches near lakes or canals, at the end of the breeding season of 1975.

Biometrics

Living frogs were measured using a vernier caliper. From each animal the following measures were taken (abbreviations and accuracies are placed between brackets): body length, tibia length (t.; 0.2 mm), length of digitus primus (d.p.; 0.3 mm), length of callus internus (c.i.l.; 0.2 mm) and height of callus internus (c.i.h.; 0.2 mm). Except, of course, body length all measures were taken on both sides of the body.

Blood sampling

Blood was taken in the field from the feet, after clipping a few toes for marking, or cutting a blood vessel between the fourth and fifth toe of a hind limb. Especially the latter manner provides a quick method to get some 20 µl of blood. After bleeding the frogs, relatively unharmed, were released. Blood was collected in glass capillaries of 0.75 mm inner diameter, containing approximately 1 µl of a 4% solution of Na-citrate

to prevent clotting. During field-work the capillaries were sealed at one end with paraffin and stored in ice-filled polystyrene boxes. Back in the laboratory the paraffin was removed, the capillaries were sealed again by melting one end in a gasflame and centrifugated (15 min., 4000 rpm). Hereafter the plasma was mixed with an equal volume of a 70% sucrose solution and stored at −25° C.

Electrophoresis

The plasma albumin pattern was studied by means of vertical polyacrylamide gel electrophoresis using the Pharmacia GE-4 apparatus containing 1 to 4 slabs with 12 samples each. The gel-system used was gel-system No. 1 by MAURER (1971). Electrophoresis was carried out with 450 V, 30 mA per slab during 90 minutes. Sample size was approximately 3 µl of plasma-sucrose mitxure. After electrophoresis gels were stained with Amido Black 10B, destained with active charcoal (GATHERCOLE & KLEIN, 1971) and stored in 7% acetic acid.

Statistics

Relative dimensions of tibia, digitus primus and callus internus are considered to be the most useful biometrical characteristics to distinguish different forms of green frogs from each other (see for instance BERGER, 1973). Therefore the ratios t./c.i.l. (index 1), d.p./c.i.l. (index 2), t./c.i.h. (index 3) and d.p./c.i.h. (index 4) were used for statistical analyses. All calculations were separately made for either side of the body. Mean values were compared by analysis of variance. By means of discriminant analysis it was investigated whether the albumin pattern can be determined from the indexes mentioned. Thereby one starts from k disjunct classes of objects (in this case 3 groups of frogs with different albumin patterns) and m measurements of each object (indexes 1 to 4), which are assumed to contain information about the class to which the object in question belongs. The result of the discriminant analysis is a criterion on the basis of the measures considered, on which the objects can be classified into k groups corresponding to the original classes. The criterion is derived on the following conditions: 1. that for each class the probability that a random drawn object belongs to that class is known (a priori probability); 2. that the simultaneous probability distribution of the measures is known. Under these conditions for each object the a posteriori probabilities are obtained that it belongs to each of the k classes and according to the criterion it is allotted to the class for which the a posteriori probability is largest. The two conditions mentioned before can never be fulfilled; for that reason one chooses reasonable values of the a priori probabilities (in this case supposed to be qual for all classes), one assumes that

the simultaneous probability distribution of the measures is of a certain type (in this case a multinormal distribution with for all classes the same covariance matrix) and one estimates the parameters of this distribution on the basis of the measures.

Under these assumptions the criterion yields k so-called classification functions, corresponding to the k classes, that are linear combinations of the measures and each object is allotted to the group for which the classification function has the largest value.

RESULTS

The serological studies revealed the presence of three different albumin patterns (Fig. 1) and thus of three frog types:

1. Type A with a fast moving albumin band;
2. Type B with a slower moving albumin band;
3. Type AB with a fast and a slower moving albumin band.

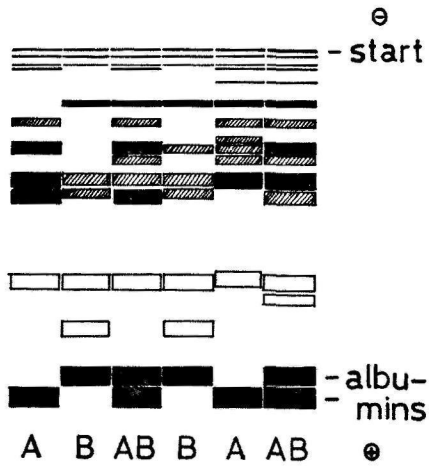

Fig. 1. The albumin pattern A, AB and B as revealed by electrophoresis.

According to the analysis of variance, each of the indexes 1 to 4 is very significantly related to the albumin patterns A, AB and B (Table I, Fig. 2). Because of the considerable overlap, it is difficult to give an accurate biometrical characterization of the frog types by using the indexes separately.

The results of the discriminant analysis, applied to the indexes 1 to 4 (I_1, I_2, I_3, I_4) are the following classification functions:

TABLE I

Results of analysis of variance for testing differences between the mean values of the indexes 1 to 4 of frogs with albumin patterns A, AB and B. I_1 = tibia length/length of callus internus, I_2 = length of digitus primus/length of callus internus, I_3 = tibia length/height of callus internus and I_4 = length of digitus primus/height of callus internus.

Index	*F-value left side of body (2, 180 DF)*	*F-value right side of body (2, 179 DF)*
I_1	402	391
I_2	192	190
I_3	320	334
I_4	262	258

p in all cases less than .01.

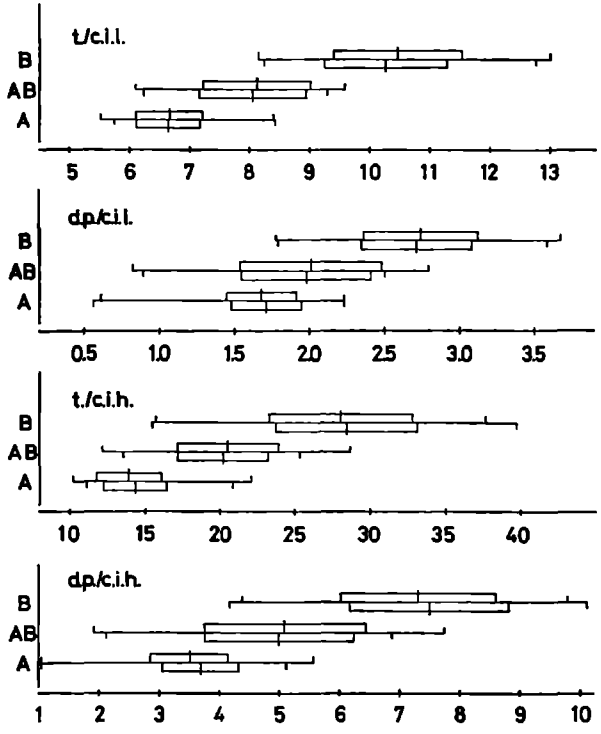

Fig. 2. Mean values, standard deviations and extreme values of the indexes 1 to 4 of frogs with albumin patterns A, AB and B. The upper half of each symbol refers to the left, the lower half to the right side of the body.

Left body side:

$$f_A = 8.1\ I_1 + 7.8\ I_2 + 2{,}8\ I_3 - 8.1\ I_4 - 38.5$$
$$f_{AB} = 9.9\ I_1 + 8.3\ I_2 + 3.7\ I_3 - 9.7\ I_4 - 61.4$$
$$f_B = 11.2\ I_1 + 15.4\ I_2 + 4.9\ I_3 - 12.9\ I_4 - 101.9$$

Right body side:

$$f_A = 6.9\ I_1 + 13.2\ I_2 + 3.2\ I_3 - 9.3\ I_4 - 39.6$$
$$f_{AB} = 8.0\ I_1 + 16.4\ I_2 + 4.4\ I_3 - 12.4\ I_4 - 62.2$$
$$f_B = 7.4\ I_1 + 30.1\ I_2 + 6.4\ I_3 - 17.7\ I_4 - 103.0$$

Applying the above functions to all frogs, 92.9 and 92.3% for the left and right body side respectively were classified correctly, i.e. were allotted to the group that corresponds with the albumin class to which they belong.

If the 4 indexes were introduced one by one (stepwise) in the discriminant analysis, according to their contribution to the classification criterion, index 1 was taken first and then index 3 and the other indexes did not yield important additional information. Consequently, applying a discriminant analysis to indexes 1 and 3 only, the classification of the frogs was almost as correct (92.9% left and 91.2% right) as for all indexes (see above). Table II presents the classification results on the basis of indexes 1 and 3. The classification functions with indexes 1 and 3 only are:

TABLE II

Classification results (expressed as numbers of frogs) on the basis of discriminant analysis applied to indexes tibia length/length of callus internus (I_1) and tibia length/height of callus internus (I_3). The uppermost number of each pair refers to the left, the other to the right body side (of one frog of albumin class B the index values of the right side of the body were missing).

Albumin class	*Predicted group*			*Total*
	A	*AB*	*B*	
A	89	3	0	92
	88	4	0	92
AB	3	22	1	26
	4	22	0	26
B	0	6	59	65
	0	8	56	64
Total	92	31	60	183
	92	34	56	182

Left body side:

$f_A = 9.2\ I_1 + 0.72\ I_3 - 35.9$
$f_{AB} = 11.0\ I_1 + 1.19\ I_3 - 56.9$
$f_B = 14.0\ I_1 + 1.68\ I_3 - 97.0$

Right body side:

$f_A = 9.9\ I_1 + 0.71\ I_3 - 37.8$
$f_{AB} = 11.7\ I_1 + 1.13\ I_3 - 58.4$
$f_B = 14.6\ I_1 + 1.69\ I_3 - 99.2$

A visualization of the biometrical characteristics of the three types of frogs is presented in figure 3 (only for the left body side).

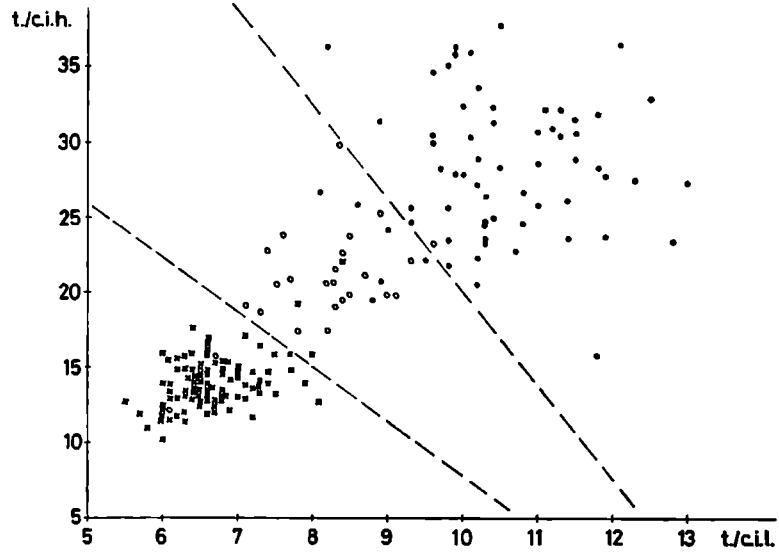

Fig. 3. Tibia length/height of callus internus plotted against tibia length/length of callus internus (only for the left side of the body) of frogs with different albumin patterns (× = A; o = AB; • = B). The lines shown are the borderlines between the groups, on the basis of the classification functions resulting from discriminant analysis.

In order to check the validity of the discriminant analysis, the criterion obtained for the left body side was applied to the index values (I_1 and I_3) of the right body side and vice versa. In both cases high percentages of frogs were classified correctly (91.2 and 91.8% respectively). If all frogs were classified according to the criterion of the left and the right body side applied to both body sides, for 22

TABLE III

Frequency distribution of misclassified frogs, according to discriminant analysis applied to I_1 and I_3. L → R means: misclassification of the criterion of the left side of the body to the measures of the right side of the body etc.

Misclassifications	*Frequency*
R → R only	1
R → L only	1
L → R only	1
L → L and R → L	4
L → R and R → R	5
L → R, R → L and R → R	1
all four	9
total	22

animals at least one misclassification occurred (Table III). Any frog that was misclassified more than once, was misclassified always in the same direction.

DISCUSSION

Because of the highly significant relation between the serological and biometrical data in our material and the strong agreement with the descriptions of the three forms of *Rana esculenta* complex given by TUNNER (1970, 1973), BERGER (1973), BLANKENHORN *et al.* (1971) and GÜNTHER (1973) it is concluded that the forms *lessonae*, *esculenta* and *ridibunda* occur in the Netherlands. For identification we think it is necessary that a frog, on the basis of its classification functions (with index 1 and index 3), is allotted to the albumin class to which it belongs. Consequently, we call a frog *lessonae* if its f_A-value is highest and if it belongs to albumin class A; a frog with the f_{AB} -value highest and albumin pattern AB is called *esculenta*; the *ridibunda* form has the F_B-value highest and albumin pattern B. In this way it was possible to identify 160 frogs (Table IV).

TABLE IV

Numbers of different forms caught in the areas studied (between brackets are placed the numbers of misclassified frogs, these are frogs which possess the albumin pattern of the form indicated, but are allotted at least once—see TABLE III—to another form on the basis of their biometrical characteristics).

Form	*Gelderland*	*Friesland*
lessonae	88 (4)	0
esculenta	20 (6)	0
ridibunda	0	52 (12)

Apart from deviations from the assumptions underlying discriminant analysis, which in this case seem to be of minor importance, and errors in the determination of the albumin pattern, which are highly improbable, the disagreement between the biometrical and serological characterization of the 22 misclassified frogs may also be due to other causes. These are errors of measurement, injuries causing anomalous body proportions or endogenous factors. That of these 22 frogs 9 were misclassified in the same direction by all classifications is likely to be attributed to endogenous factors.

In the literature (for instance BERGER, 1973) the indexes t./c.i.l. and d.p./c.i.l. are considered to be the most useful biometrical features to distinguish the forms of *Rana esculenta* complex. According to this study it seems that t./c.i.l. and t./c.i.h. are the most discriminating indexes; the latter index, however, has never been suggested by any author up to now.

Besides the features mentioned above, also other ones are used for identifying green frogs, like head shape, shape of the callus internus and body colour (TUNNER, 1970, 1973; BERGER, 1973; BLANKENHORN *et al.*, 1971 and GÜNTHER, 1973). If it should be possible to take reproducible measurements of these properties, one could perhaps achieve a better classification by means of discriminant analysis than the one presented in this paper.

TUNNER (1973) finds three albumin bands within *Rana esculenta* complex: *Rana lessonae* has only the fastest moving band, *Rana ridibunda* one or both of the other bands, resulting in three different *ridibunda* albumin patterns. He finds two *esculenta* albumin patterns, consisting of the *lessonae* band and one of the two *ridibunda* bands. According to HEMMER (1973) the slower *ridibunda* band belongs to *Rana ridibunda perezi* and the other one to *Rana ridibunda ridibunda*. In our material we only find one *ridibunda* band. For lack of comparison with *ridibunda* samples from other parts of Europe at this moment, it is not certain whether this is the band of *Rana ridibunda ridibunda* or *Rana ridibunda perezi*. However, in view of their geographical distribution one would expect to find *Rana ridibunda ridibunda* in our material.

The taxonomy and phylogeny of the European green frogs is still not fully understood. At the moment we are paying attention especially to the reproductive relationships of the different forms, by means of cross-breeding experiments and ecological and ethological investigations.

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REFERENCES

BERGER, L., 1968. Morphology of the F_1 generation of various crosses within *Rana esculenta* complex.—Acta Zool. Crac. **13:** 301–324.

—, 1970. Some characteristics of the crosses within *Rana esculenta* complex in postlarval development.—Ann. Zool. **27:** 373–416.

—, 1971a. Viability, sex and morphology of F_2 generation within forms of *Rana esculenta* complex.—Zool. Poloniae **21:** 345–393.

—, 1971b. Inheritance of sex and phenotype in F_1 and F_2 crosses within *Rana esculenta* complex.—Genetica Poloniae **21:** 517–521.

—, 1973. Systematics and hybridization in European green frogs of *Rana esculenta* complex.—J. Herpetol. **7:** 1–10.

—, & MICHALOWSKI, J., 1971. Translation of "Keys for the identification of Vertebrates of Poland". Part 2 Amphibia.—Scientific Publications Foreign Cooperation Center of the Central Institute for Scientific, Technical and Economic Information, Warsaw.

BLANKENHORN, H. J., H. HEUSSER & P. VOGEL, 1971. Drei Phanotypen von Grunfroschen aus dem *Rana esculenta*-Komplex in der Schweiz.—Rev. Suisse Zool. **78:** 1243–1247.

BOULENGER, G. A., 1891. A contribution to the knowledge of the races of *Rana esculenta* and their geographical distribution.—Proc. Zool. Soc. London **1891:** 374–384.

—, 1918. On the races and variation of the edible frog, *Rana esculenta* L.—Ann. Mag. Nat. Hist. **2:** 241–257.

GATHERCOLE, L. J. & L. KLEIN, 1971. Simple device for continuous diffusion destaining of polyacrylamide gels.—Anal. Biochem. **44:** 232–234.

GELDER, J. J. VAN, 1973. A quantitative approach to the mortality resulting from traffic in a population of *Bufo bufo* L.—Oecologia (Berl.) **13:** 93–95.

—, & HOEDEMAEKERS, H. C. M., 1971. Sound activity and migration during the breeding period of *Rana temporaria* L., *R. arvalis* Nilsson, *Pelobates fuscus* Laur. and *Rana esculenta* L.—J. Anim. Ecol. **40:** 559–568.

GÜNTHER, R., 1968. Morphologische und okologische Untersuchungen zur Unterscheidung von *Rana esculenta* L. und *Rana ridibunda* Pall.—Zool. Jb. Syst. **95:** 229–264.

—, 1970. Der Karyotyp von *Rana ridibunda* Pall. und das Vorkommen von Triploidie bei *Rana esculenta* L. (Anura, Amphibia).—Biol. Zentralbl. **89:** 327–342.

—, 1973. Über die verwandtschaftlichen Beziehungen zwischen den europaischen Grunfroschen und den Bastardcharakter von *Rana esculenta* L. (Anura).—Zool. Anz. **190:** 250–285.

HEMMER, H., 1973. Das Serumeiweissbild von *Rana ridibunda perezi* im Rahmen des *Rana esculenta-lessonae-ridibunda*-Komplexes (Salientia, Ranidae).—Salamandra **9:** 168–172.

HOOGMOED, M. S., 1975. On the presence of *Rana ridibunda ridibunda* Pallas in the Netherlands.—Zool. Bijdr. Leiden **17:** 72–76.

KARAMAN, S., 1948. Prilog herpetologiji sjeverne Srbije.—Prirodosl. istraž., Zagreb **24:** 51–73.

KAURI, H., 1954. Über die systematische Stellung der europaischen grunen Frosche *Rana esculenta* L. und *Rana ridibunda* Pall.—Kungl. Fysiogr. Sallsk. Handl. **65:** 1–30.

—, 1959. Die Rassenbildung bei europaischen *Rana*-Arten und die Gültigkeit der Klimaregeln.—Ann. Soc. tart. Res. nat. Invest. Const., Ser. nov., Lund **2:** 1–172.

MAURER, H. R., 1971. Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. De Gruyter, New York.

MERTENS, R. & H. WERMUTH, 1960. Die Amphibien und Reptilien Europas. Waldemar Kramer, Frankfurt am Main.

TUNNER, H. G., 1970. Das Serumeiweissbild einheimischer Wasserfrosche und der Hybridcharakter von *Rana esculenta*.—Verh. dtsch. zool. Ges. **64:** 352–358.

—, 1972. Serologische und morphologische Untersuchungen zur Frage der Artabgrenzung bei Wasserfroschen aus der Umgebung von Mainz (Rhein-Main-Gebiet).—Z. zool. Syst. Evolut. forsch. **10:** 127–132.

—, 1973. Das Albumin und andere Bluteiweisse bei *Rana ridibunda* Pallas, *Rana lessonae* Camerano, *Rana esculenta* Linné und deren Hybriden.—Z. zool. Syst. Evolut. forsch. **11:** 219–233.

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Plasma Albumins and Biometrical Characteristics of Different Forms of *Rana esculenta* Complex

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With one figure

Abstract

Within the scope of a study of *Rana esculenta* complex biometrical and serological characteristics of 523 green frogs from different parts of France and the Netherlands were examined. Attention was especially directed at *Rana ridibunda perezi*, since in literature conflicting information is given as to the plasma albumin pattern of this subspecies.

Both in France and in the Netherlands 4 different albumin patterns were found, one of *Rana lessonae* (A), one of *Rana esculenta* (AB) and two of *Rana ridibunda* (B and BC). However, none of the supposed albumin patterns of *Rana ridibunda perezi* was found. There were no significant differences between the two albumin types of *ridibunda* collected in France, as regards to the biometrical characteristics studied.

Only of albumin type B sufficient samples were available for a meaningful comparison of frogs from both countries. In this case significant biometrical differences appeared to exist, which found expression in the numbers of frogs classified correctly on the basis of discriminant analysis: in the Netherlands a significantly lower number of frogs was classified correctly than in France. This might be related to the sympatrical occurrence of *lessonae*, *esculenta* and *ridibunda* in the Netherlands, all of the French *ridibunda* originated from presumably pure populations from various parts of the Mediterranean region.

I. Introduction

Since Berger started his research on European green frogs (*Rana esculenta* complex) in Poland, many biologists — especially in Central Europe — have tackled the problems concerning this interesting systematical group. Apart from morphological studies and cross-breeding experiments (carried out by most investigators), special attention was paid to serological (Tunner 1970, 1972 and 1973) and enzymological aspects (Uzzell and Berger 1975, Günther and Hahnel 1976, Tunner and Dobrowsky 1976, Vogel and Chen 1976). All these efforts resulted in the hypothesis — which is nowadays almost generally accepted — that *Rana lessonae* and *Rana ridibunda* are distinct species, whereas *Rana esculenta* is their hybrid (see Hotz 1974 for a review of the most important literature up to 1974).

Strangely enough, in the western parts of Europe hardly any investigations have been made of this subject, whereas especially France seems worth while because of its size and because of the occurrence of *Rana ridibunda perezi* Seoane 1885 in the Mediterranean region and of *Rana ridibunda ridibunda* Pallas 1771 and *Rana esculenta* Lin-

NAEUS 1758 in the northern parts (see MERTENS and WERMUTH 1960). Clear information is lacking concerning the presence of *Rana lessonae* CAMERANO 1882 in this country.

In 1976 WIJNANDS and VAN GELDER published biometrical and serological data by which they were able to prove that *Rana lessonae*, *Rana esculenta* and *Rana ridibunda* occur in the Netherlands. Unfortunately, by lack of the necessary information, the subspecific status of the *Rana ridibunda* studied could only be deduced from its geographical distribution, a rather risky criterion. For that reason it was decided to examine biometrical and serological samples from different parts of France and to compare these with data gathered in the Netherlands. Another aim of this study was to end the confusion caused by the conflicting information given by HEMMER (1973) and TUNNER and UZZELL (1974) concerning the plasma albumin of *Rana ridibunda perezi*.

II. Material and Methods

1. *Collecting stations*

In France samples were taken during two 3-week journeys which were also made for other purposes.

In August and September 1975 frogs were caught at the following places:

1) Lac de la Liez, a large artificial lake near the city of Langres (Haute-Marne).
2) Villeneuve, a hamlet in the Camargue (Bouches-du-Rhône). Here frogs were caught in ditches.
3) Le Sambuc, see Villeneuve.
4) Baillaury, a small river running through the village of Banyuls-sur-Mer (Pyrénées-Orientales).
5) Tech, a medium-sized river in Pyrénées-Orientales. Frogs were caught near the town of Elne.
6) Etang de Lers, a lake at an altitude of about 1300 metres near the village of Aulus-les-Bains (Ariège).

In September 1976 frogs were caught in the:

7) Môle, a small river in Var. Frogs were caught between the village of La Môle and the "Col de Gratteloup".
8) Orb, a medium-sized river running through the city of Béziers (Hérault). Frogs were caught near the village of Cessenon.

The Dutch material originated from the biometrical and serological investigations by WIJNANDS and VAN GELDER (1976) and from a study of the distribution and habitat of *Rana esculenta* complex in the Netherlands, which has not been published yet.

2. *Biometrics, Blood Sampling and Electrophoresis*

Apart from body length the following measures were taken (in 0.1 mm, on both sides of the body): tibia length, length of digitus primus, length and height of callus internus. Only adult frogs were measured.

In the field some blood was taken from a blood vessel between the fourth and fifth toe of a hindlimb. It was centrifuged immediately in a hand centrifuge whereafter the plasma was stored at 0 °C during the journeys, adding Merthiolate (Lilly and Co.) in a final concentration of 1: 10,000 as a preservative. Immediately after returning in the laboratory at Nijmegen it was frozen at −20 °C.

Vertical polyacrylamide gel electrophoresis was applied to analyse the albumin pattern.

More detailed information about the above methods can be found elsewhere (WIJNANDS and VAN GELDER 1976).

3. *Statistics*

Relative dimensions of tibia, callus internus and, to a lesser degree, digitus primus yield very useful criteria to distinguish different froms of green frogs from each other. Therefore, biometrical

differences between frogs with different albumin patterns and from different places were tested by applying analysis of variance or t-tests to the following indices:
I_1 = tibia length/length of callus internus;
I_2 = length of digitus primus/length of callus internus;
I_3 = tibia length/height of callus internus;
I_4 = length of digitus primus/height of callus internus.
Either side of the body was examined separately. In addition discriminant analysis was carried out (see WIJNANDS and VAN GELDER 1976); the resultant classification functions were applied to the corresponding body side only.

III. Results

Apart from the albumin patterns (A, AB and B) which were already known for the Netherlands (WIJNANDS and VAN GELDER 1976), the electrophoresis revealed another type. It consists of two bands, the faster of which moves at the same speed as the band of albumin pattern B, the other one slightly slower (Fig. 1, albumin pattern BC). The index values belonging to this albumin pattern do not differ significantly from those of type B (t-Student or t-Welch, level of significance = .05). Other differences between albumin patterns were not tested. The results of tests of differences between the main study areas (France, Friesland, the Netherlands exclusive of Friesland) can be found in Table 1, which shows also biometrical parameters and sample sizes for the complete material.

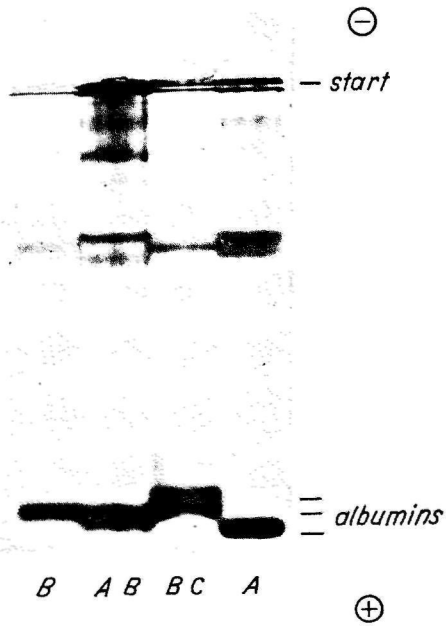

Fig. 1. Electropherograms of blood plasma from frogs with different albumin patterns (A, AB, B and BC).

Table 1. Means, standard deviations, sample sizes and statistical tests of indices 1 to 4 of frogs with different albumin patterns and from different places. I_1 — tibia length/length of callus internus, I_2 — length of digitus primus/length of callus internus, I_3 — tibia length/height of callus internus I_4 — length of digitus primus/height of callus internus

Index	Albumin	Means ± standard deviations and sample sizes (in brackets) for the left (upper number) and right (lower number) body side. Significant differences within rows are indicated by similar symbols. Level of significance .05: ? = .05 < p < .10			Statistical test □ A.V. — analysis of variance
I_1		France	Friesland*	The Netherlands**	
	A	7.7 (1)	—	6.23 ± 0.63 (31)	—
		8.0 ± 0.4 (2)		6.24 ± 0.59 (31)	—
	AB	9.02 ± 0.69 (30) ●	-	7.56 ± 0.66 (150) ●	t-Student
		9.12 ± 0.70 (30) ●	—	7.54 ± 0.62 (151) ●	t-Student
	B	11.22 ± 1.13 (139) ○	10.46 ± 1.07 (65) ○ ▲	9.70 ± 1.03 (81) ▲ ⨯	A.V. + Scheffé-test
		11.39 ± 1.24 (137) ⨯ ○	10.27 ± 1.03 (64) ○ ?	9.83 ± 0.97 (83) ? ⨯	A.V. + Scheffé-test
	BC	12.05 ± 2.09 (13)	—	11.9 ± 0.1 (2)	—
		12.07 ± 1.92 (13)	—	11.4 ± 1.1 (2)	—
I_2					
	A	2.0 (1)	—	1.71 ± 0.23 (31)	-
		2.1 ± 0.1 (2)	-	1.71 ± 0.20 (30)	-
	AB	2.37 ± 0.30 (30) ●		2.07 ± 0.30 (147) ●	t-Student
		2.44 ± 0.24 (30) ●	—	2.07 ± 0.30 (148) ●	t-Student
	B	2.92 ± 0.33 (139) ⨯ ○	2.74 ± 0.39 (64) ○ ?	2.61 ± 0.35 (88) ? ⨯	A.V. + Scheffé-test
		2.98 ± 0.36 (138) ○	2.71 ± 0.37 (63) ○	2.62 ± 0.33 (87) ⨯	A.V. + Scheffé-test
	BC	3.07 ± 0.48 (13)	—	3.5 ± 0.2 (2)	-
		2.95 ± 0.39 (13)	—	3.3 ± 0.6 (2)	—

I_q	France	Friesland*	The Netherlands**	
A	13.1 (1)	—	13.99 ± 2.59 (31)	—
	14.4 ± 1.8 (2)	—	13.39 ± 1.84 (31)	—
AB	20.44 ± 2.57 (30) ●	—	18.68 ± 2.79 (150) ●	t-Student
	20.61 ± 2.49 (29) ●	—	18.42 ± 2.86 (151) ●	t-Student
B	26.14 ± 3.76 (139) × ○	28.12 ± 4.72 (65) ○ ?	29.76 ± 4.35 (89) ? ×	A.V. — Scheffé-test
	26.20 ± 3.36 (136) × ○	28.46 ± 4.66 (65) ○	28.97 ± 3.80 (88) ×	A.V. + Scheffé-test
BC	25.32 ± 3.54 (13)	—	28.4 ± 1.1 (2)	—
	27.09 ± 3.40 (13)	—	29.7 ± 4.5 (2)	—
I_1				
A	3.5 (1)	—	3.78 ± 0.82 (31)	—
	3.8 ± 0.4 (2)	—	3.65 ± 0.55 (30)	—
AB	5.35 ± 0.75 (30)	—	5.11 ± 0.99 (147)	t-Student
	5.51 ± 0.68 (29) ●	—	5.05 ± 1.04 (148) ●	t-Welch
B	6.82 ± 1.06 (139) × ○	7.33 ± 1.28 (64) ○ ▲	7.90 ± 1.25 (88) ▲ ×	A.V. + Scheffé-test
	6.87 ± 0.95 (137) × ○	7.50 ± 1.33 (64) ○	7.65 ± 1.05 (87) ×	A.V. + Scheffé-test
BC	6.48 ± 0.89 (13)	—	8.4 ± 0.8 (2)	—
	6.62 ± 0.80 (13)	—	8.4 ± 0.7 (2)	—

*see WIJNANDS and VAN GELDER (1976)
**exclusive of the data from Friesland

Table 2. Classification results (expressed as numbers of frogs and percentages) after application of the classification functions (with I_1 and I_3) from Wijnands and van Gelder (1976) to all frogs. The percentages have been calculated exclusive of albumin class BC, which has not been used in the formation of the classification functions. Only the results for the left body side are shown

Albumin class	Predicted group			Total
	A	AB	B	
A	31	1	0	32
	96.9 %	3.1 %	0.0 %	
AB	50	129	1	180
	27.8 %	71.7 %	0.6 %	
B	0	29	264	293
	0.0 %	9.9 %	90.1 %	
BC	0	0	15	15
Total	81	159	280	520

Overall percentage of frogs classified correctly: 84.0 %

Application of the classification functions (with indices 1 and 3 only) resulting from a discriminant analysis in a previous study (Wijnands and van Gelder 1976) yields high overall percentages of frogs classified correctly (i. e. allotted to the albumin class to which they belong, both body sides considered separately), as is shown in Table 2. Note that all frogs with albumin BC, which has not been used in the formation of the classification functions, are allotted to albumin class B. The overall percentages of frogs classified correctly after a new discriminant analysis (including albumin BC) are relatively low, which is mainly caused by allocation of frogs from albumin B to BC and vice versa (see Table 3).

Table 3. Classification results (expressed as numbers of frogs and percentages) according to discriminant analysis including albumin class BC (left body side only). As in this analysis I_4 was selected in addition to I_1 and I_3, the numbers of frogs do not match those presented in Table 2, due to occasionally missing values

Albumin class	Predicted group				Total
	A	AB	B	BC	
A	30	2	0	0	32
	93.8 %	6.3 %	0.0 %	0.0 %	
AB	24	148	4	1	177
	13.6 %	83.6 %	2.3 %	0.6 %	
B	0	15	199	77	291
	0.0 %	5.2 %	68.4 %	26.5 %	
BC	0	0	5	10	15
	0.0 %	0.0 %	33.3 %	66.7 %	
Total	54	165	208	88	515

Overall percentage of frogs classified correctly: 75.1 %

The distribution of the albumin patterns over the collecting stations in France is presented in Table 4. It should be noted that at collecting station 6 only older and already metamorphosing larvae (stages 29—32, WITSCHI 1956) were found.

Table 4. Frequency distribution of the albumin patterns A, AB, B and BC over the collecting stations in France

Collecting station	Albumin pattern			
	A	AB	B	BC
1) Lac de la Liez	2	30	—	—
2) Villeneuve	—	—	24	—
3) Le Sambuc	—	—	7	—
4) Baillaury	—	—	60	13
5) Tech	—	—	10	—
6) Etang de Lers	—	—	—	22*
7) Môle	—	—	16	—
8) Orb	—	—	23	—
Total	2	30	140	35

* only larvae

IV. Discussion

The total number of albumin patterns that have been found in European green frogs up to now amounts to 7 : 1 of *Rana lessonae* (Al/Al), 3 of *Rana ridibunda ridibunda* (Ar_1/Ar_1, Ar_2/Ar_2, Ar_1/Ar_2), 2 of *Rana esculenta* (Al/Ar_1, Al/Ar_2), all found by TUNNER (1973), and 1 of *Rana ridibunda perezi* (TUNNER and UZZELL 1974). The occurrence of three of these in the Netherlands has already been established, but it was impossible to identify the *ridibunda* band at that moment (WIJNANDS and VAN GELDER 1976). The results presented in this paper allow the following statements: albumin A corresponds with Al/Al founds by TUNNER (1973); AB is identical with Al/Ar_1 (TUNNER 1973); B and BC correspond with Ar_1/Ar_1, and Ar_1/Ar_2 (TUNNER 1973) respectively. There are significant biometrical differences between *lessonae*, *esculenta* and *ridibunda*, but not between frogs (*ridibunda*) with albumin pattern B or BC, at least not as regards to the indices studied here. All of the four albumin patterns mentioned occur both in France and in the Netherlands, although the origin of albumin BC (only two frogs) in the Netherlands is doubtful. It is true that of albumin A in France an equally low number has been found, but this is probably a consequence of the incidental sampling.

The uncertainty concerning the albumin pattern of *Rana ridibunda perezi* still remains. According to HEMMER (1973) this form possesses a single albumin band corresponding with albumin pattern Ar_2/Ar_2 found by TUNNER (1973); in our notation it would have been called C. TUNNER and UZZELL (1974), however, state that the albumin pattern of *Rana ridibunda perezi* consists of a single band, lying between Al/Al (the essonae band, A in our notation) and Ar_1/Ar_1 (the fastest band of *Rana ridibunda ridi-*

Table 5. Numbers of frogs with albumin pattern B, classified correctly at both sides of the body according to the classification functions (with I_1 and I_3) from WIJNANDS and VAN GELDER (1976). Differences between the main study areas are tested by Chi²-tests

	France	Friesland	The Netherlands*
classified correctly	127	53	71
misclassified	8	11	18

$Chi^2 = 10.90$; df = 2; p = .004, significant at .05 level

2×2-tests: (df = 1)	Chi²	p
France-Friesland	5.14	.023 n.s.
France-The Netherlands	9.34	.004 s.
Friesland-The Netherlands	0.07	.79 n.s.

*exclusive of Friesland

n.s. = not significant; s. = significant; both at .05/3 level = simultaneously at .05 level.

bunda, in our notation B). Unfortunately, these three authors do not explain why their frogs belong to the subspecies *perezi*. Besides, TUNNER and UZZELL (1974) give only vague indications of the origin of the frogs they studied, whereas HEMMER (1973) examined but three specimens. In fact, there is no evidence of a significant relation between the morphological subspecies *Rana ridibunda perezi* SEOANE 1885 and any particular plasma albumin pattern. The results of the present study only seem to increase the confusion instead of ending it, as none of the possible *perezi* patterns mentioned above was found in the south of France, which according to MERTENS and WERMUTH (1960) lies within the distribution area of the subspecies *perezi*. It is true that the albumin band which HEMMER (1973) says to belong to *perezi* is present in the samples studied, but only in combination with another *ridibunda* band, together forming albumin pattern BC. Any further discussion of this problem seems to be senseless at this moment, as the necessary basic information is lacking.

The biometrical differences between frogs from the three main study areas (France; Friesland; the Netherlands exclusive of Friesland) may have several causes (only the data of frogs with albumin pattern B will be discussed since only of these sufficient samples were available). In the first place the way of measuring may be changeable, even with the same investigator. This brings on deviations which probably are small but can never be excluded. Secondly, the biometrical differences observed may be due to geographical and ecological differences between the study areas and are as such not typical of *Rana esculenta* complex. However, it is also possible that the sympatrical occurrence of *lessonae*, *esculenta* and *ridibunda* in the Netherlands allows gene flow between the three forms and consequently a nivellation of some of the differences between them; this would explain why in the Netherlands fewer frogs of albumin class B were classified correctly than in (the south of) France where only *ridibunda* (mainly albumin B) was found (see Table 5). However, this suggestion would conflict with the hypothesis by TUNNER (1974) that the maintenance of the hybrid-status of *esculenta* results from hybridogenesis, which does not allow much gene flow between *lessonae* and *ridibunda*.

As regards to the occurrence of the different forms at the collecting stations it seems worth mentioning that in the south of France *ridibunda* was found in all sorts of waters, in contrast with the general picture which in the literature is given of the habitat of this form in Central Europe (Berger 1973. Günther 1974, Tunner and Dobrowski 1976). In the Netherlands *ridibunda* inhabits different types of waters too. This will be shown in another paper, which deals with the distribution and habitat of *Rana esculenta* complex in the Netherlands.

Zusammenfassung

Im Rahmen einer Studie des *Rana esculenta*-Komplexes wurden biometrische und serologische Merkmale von 523 Grünfröschen aus verschiedenen Teilen Frankreichs und den Niederlanden untersucht. Besondere Aufmerksamkeit wurde *Rana ridibunda perezi* geschenkt, weil über das Plasmaalbumin dieser Subspezies in der Literatur widersprüchliche Daten gemeldet werden.

Sowohl in Frankreich wie in den Niederlanden wurden 4 verschiedene Albuminmuster gefunden, eins von *Rana lessonae* (A), eins von *Rana esculenta* (AB) und 2 von *Rana ridibunda* (B und BC). Es wurde aber keines der gemeldeten Albuminmuster von *Rana ridibunda perezi* gefunden. Hinsichtlich der untersuchten biometrischen Merkmale gab es keine signifikanten Unterschiede zwischen den beiden Albumintypen von *ridibunda* aus Frankreich.

Für einen sinnvollen Vergleich zwischen Froschen aus beiden Ländern waren nur ausreichende Anzahlen des Albumintyps B vorhanden. Hierbei gab es signifikante Unterschiede in der Anzahl von Froschen, die mit der Diskriminanzanalyse eingeteilt werden konnten: in den Niederlanden konnten weniger Frösche sicher zugeordnet werden als in Frankreich. Dies dürfte auf das sympatrische Vorkommen von *lessonae*, *esculenta* und *ridibunda* in den Niederlanden zurückzuführen sein, während alle französischen *ridibunda* aus verschiedenen Teilen des Mittelmeergebietes aus vermutlich reinen Populationen stammen.

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Literature

Berger, L.: Systematics and hybridization in European green frogs of *Rana esculenta* complex. J. Herpetol. **7** (1973), 1–10.

Günther, R.: Neue Daten zur Verbreitung und Ökologie der Grünfrosche (Anura, Ranidae) in der DDR. Mitt. Zool. Mus. Berlin **50** (1974), 287–298.

— und Hahnel, S.: Untersuchungen über den Genfluß zwischen *Rana ridibunda* und *Rana lessonae* sowie die Rekombinationsrate bei der Bastardform *Rana „esculenta"* (Anura, Ranidae). Zool. Anz. **197** (1976), 23–38.

Hemmer, H.: Das Serumeiweißbild von *Rana ridibunda perezi* im Rahmen des *Rana esculenta-lessonae-ridibunda*-Komplexes (Salientia, Ranidae). Salamandra **9**, (1973) 168–172.

Hotz, H.: Ein Problem aus vielen Fragen — europaische Grünfrosche (*Rana esculenta*-Komplex) und ihre Verbreitung. Natur und Museum **104** (1974), 262–272.

Mertens, R., und Wermuth, H.: Die Amphibien und Reptilien Europas. Frankfurt am Main 1960.

Tunner, H. G.: Das Serumeiweißbild einheimischer Wasserfrösche und der Hybridcharakter von *Rana esculenta*. Verh. dtsch. zool. Ges. **64** (1970), 352—358.

— Serologische und morphologische Untersuchungen zur Frage der Artabgrenzung bei Wasserfröschen aus der Umgebung von Mainz (Rhein-Main-Gebiet). Z. zool. Syst. Evolut.forsch. **10** (1972), 127—132.

— Das Albumin und andere Bluteiweiße bei *Rana ridibunda* Pallas, *Rana lessonae* Camerano, *Rana esculenta* Linné und deren Hybriden. Z. zool. Syst. Evolut.forsch. **11** (1973), 219—233.

— Die klonale Struktur einer Wasserfroschpopulation. Z. zool. Syst. Evolut.forsch. **12** (1974), 309 bis 314.

— und Dobrowski, M.-Th.: Zur morphologischen, serologischen und enzymologischen Differenzierung von *Rana lessonae* und der hybridogenetischen *Rana esculenta* aus dem Seewinkel und dem Neusiedlersee (Österreich, Burgenland). Zool. Anz. **197** (1976), 6—22.

— und Uzzell, Th.: Das Serumalbumin bei *Rana ridibunda perezi* (Salientia, Ranidae). Salamandra **10** (1974), 137—139.

Uzzell, Th., and Berger, L.: Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae* and their hybridogenetic associate, *Rana esculenta*. Proc. Acad. Nat. Sci. Philadelphia **127** (1975), 13—24.

Vogel, P., and Chen, P. S.: Genetic control of LDH isozymes in the *Rana esculenta complex*. Experientia **32** (1976), 304—307.

Witschi, E.: Development of Vertebrates. Philadelphia 1956.

Wijnands, H. E. J., and van Gelder, J. J.: Biometrical and serological evidence for the occurrence of three phenotypes of green frogs (*Rana esculenta* complex) in the Netherlands. Neth. J. Zool. **26** (1976), 414—424.

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DISTRIBUTION AND HABITAT OF RANA ESCULENTA COMPLEX IN THE NETHERLANDS

by

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SUMMARY

In various waters distributed widely over the Netherlands green frogs were collected in order to get a picture of the distribution and habitat of the different forms belonging to *Rana esculenta* complex. The distinction between these forms—*lessonae*, *esculenta* and *ridibunda*—was based on biometrical and serological characteristics.

Apart from the albumin patterns which were already known for the Netherlands—A (*lessonae*), AB (*esculenta*) and B (*ridibunda*)—a new type was found, viz. albumin pattern BC belonging to *ridibunda*.

Especially many frogs with albumin pattern AB were misclassified (*i.e.* allotted to another albumin class) by means of discriminant analysis, on the basis of their biometrical characteristics. This cannot be explained at the moment.

Ridibunda was mainly found in areas abounding in water lying in the northern half of the country, but the type and size of the water seems to be of minor importance. *Lessonae* lives in smaller, often isolated waters, probably in most parts of the country. *Esculenta* can be found in all sorts of waters throughout the country, often together with one, seldom both, of the other forms.

INTRODUCTION

In the Netherlands three forms of green frogs (*Rana esculenta* complex) occur: *lessonae*, *ridibunda* and *esculenta* (WIJNANDS & VAN GELDER, 1976). Of these, the former two seem to be valid species, whereas *esculenta* is considered to be their hybrid (BERGER, 1973; GÜNTHER, 1973; TUNNER, 1973; HOTZ, 1974).

In recent years most investigations of the systematical relationships of the different forms focussed on genetical problems (UZZELL & BERGER, 1975; GÜNTHER & HÄHNEL, 1976; TUNNER & DOBROWSKY, 1976; VOGEL & CHEN, 1976). Only few investigators (*e.g.* GÜNTHER, 1974; HEYM, 1974) have published detailed data about the ecology of *lessonae*, *esculenta* and *ridibunda*.

In this paper results are presented of a study of the distribution and habitat of *Rana esculenta* complex in the Netherlands, as a basis for further ecological examinations.

MATERIALS AND METHODS

Collecting stations

In order to get a reliable picture of the distribution of the different forms, collecting stations were chosen that were distributed widely over the country. Brief descriptions of the environment in which frogs were found were made to get an impression of their habitat. Frogs were caught in 1976 at the following places (ranged in chronological order) (see Fig. 2):

1) Three adjacent ponds with rather steep slopes and only little border vegetation of *Phragmites* and *Typha*, surrounded by birch and firwood, arable land and buildings, situated in the village of Schinveld (in the South of the Province of Limburg) (May 5).
2) Two fens close to the „Rode Beek" (transl. Red Brook) in the „Brunssummerheide", a moorland en firwood area near the town of Brunssum (in the South of the Province of Limburg). The fens contained a dense vegetation of *Phragmites* and many branches of trees and shrubs (May 6).
3) The „Heerenven", a fen of very variable water level and consequently changing border vegetation (*Scirpus*, *Carex*, *Juncus*, *Calluna*, *Molinia* and even *Pinus*), situated in the moorland and firwood area „De Hamert" (in the North of the Province of Limburg). See also Van Gelder & Oomen (1970) (May 11).
4) A pool with some *Juncus* vegetation, surrounded by trees and shrubs, in a former sand-pit near „De Hamert" (see 3) (May 11).
5) A ditch with only grass-grown borders, along a road („Twistedenerweg") near „De Hamert" (see 3) (May 11).
6) An excavated pool full of *Stratiotes*, with a dense border vegetation of herbs, grasses, shrubs and trees, in the swamp area 'De Wieden' near the village of Zwartsluis (in the North of the Province of Overijssel) (May 23).
7) Several ditches and pools in the State Reserve 'De Weerribben', another swamp area in the North of the Province of Overijssel (May 25).
8) An excavated pool full of *Stratiotes*, with a dense border vegetation of herbs, grasses and shrubs, close to the 'Linde', a small river near the village of Wolvega (in the South of the Province of Friesland) (May 26).
9) Two adjacent fens surrounded by heather in a firwood and moorland area near the village of Wilhelminaoord (in the West of the Province of Drenthe) (July 8).
10) A few pools close to each other and to the river IJssel, about 2 kilometres North of the village of Terwolde (Province of Over-

ijssel). The shores of the pools were fully overgrown by herbs, grasses, shrubs and trees and the water contained much *Nymphoides* and *Nuphar* (July 23).

11) Two ditches in a meadow along a road between the villages of 's-Graveland and Ankeveen (Province of Noord-Holland), containing much *Ceratophyllum* and with a border vegetation of *Phragmites* and *Acorus* (July 29).
12) A pool with shores fully overgrown by herbs, shrubs and trees, belonging to 'De Moerkuilen', a cluster of pools surrounded by woods near the village of St. Oedenrode (Province of Noord-Brabant) (August 4).
13) A ditch with grass-grown borders, with arable land and meadows with scattered bushes on both sides, between the villages of Mariahout and Lieshout (Province of Noord-Brabant) (August 4).
14) A small and rather polluted river called 'Kleine Dommel'; frogs were caught near a bridge close to the village of Heeze (Province of Noord-Brabant) (August 4).
15) A few ditches with only little vegetation of higher waterplants, in the polders between the villages of Edam and Warder (Province of Noord-Holland) (August 11).
16) A pool in a waste land area in the village of Monnickendam (Province of Noord-Holland) (August 11).
17) A pond almost without border vegetation, in the polder of 'De Breede Watering' to the West of the town of Goes (Province of Zeeland (September 15).

The distinction between the frog types

Plasma albumin patterns and biometrical characteristics (of adult frogs only) were used to distinguish the different forms of green frogs from each other. Polyacrylamide gel electrophoresis was applied to analyse the albumin patterns. The biometrical characteristics were evaluated by means of the classification functions with I_1 and I_3, presented by WIJNANDS & VAN GELDER (1976). These functions, in which I_1 and I_3 stand for the ratios tibia length/length of callus internus, and tibia length/height of callus internus, respectively, were obtained by discriminant analysis. In the above-mentioned paper details of these methods can be found. In the present study the classification functions (which are slightly different for both body sides) are applied to the measurements of the corresponding body side only.

RESULTS

In addition to the albumin patterns that were already known for the Netherlands, namely A (*lessonae*), AB (*esculenta*) and B (*ridibunda*) (WIJNANDS & VAN GELDER, 1976) another type was found, called BC (Fig. 1). Table I shows the frequencies of these albumin patterns in the samples from the different collecting stations. The results of the biometrical examinations are presented in Table II as the numbers of frogs that were classified correctly (*i.e.* were allotted to the albumin class to which they belong) by application of the classification functions and frogs that were misclassified on the basis of the values of the measurements of at least one body side.

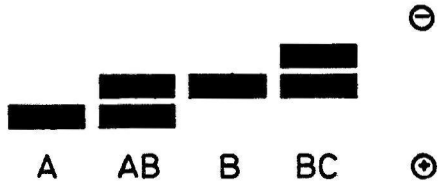

Fig. 1. Schematic drawing of albumin pattern A, AB, B and BC.

TABLE I

Frequency distribution of albumin pattern A, AB, B and BC over the collecting stations. The numbers in brackets refer to first-year juveniles, the other ones to adult frogs

collecting station	*albumin pattern*			
	A	AB	B	BC
1		21		
2	5	8		
3	2	2		
4	5	13		
5		19		
6			37	
7			8	
8		1	3	
9	5	21		
10		18		
11			8(7)	2(5)
12		3		
13	11	13		
14	3	14	5	
15		8	7	
16			21	
17		10(6)		
Total	31	151(6)	89(7)	2(5)

TABLE II

Numbers of frogs that were classified correctly (*i.e.* were allotted to the albumin class to which they belong, on the basis of their biometrical characteristics; 'correct') and frogs that were misclassified (*i.e.* were allotted to another albumin class on the basis of the values of the measurements of at least one body side; 'wrong'). Albumin class BC has been omitted (only 2 frogs)

collecting station	*albumin A*		*albumin AB*		*albumin B*	
	correct	*wrong*	*correct*	*wrong*	*correct*	*wrong*
1			1	20		
2	5	0	2	6		
3	2	0	2	0		
4	5	0	5	8		
5			9	10		
6					31	6
7					8	0
8			1	0	1	2
9[1]	4	1	15	5		
10			15	3		
11					7	1
12			2	1		
13	11	0	12	1		
14	3	0	10	4	2	3
15			4	4	4	2
16					18	4
17			10	0		
Total	30	1	88	62	71	18

[1] One frog with albumin pattern AB has been omitted as its left body side values were missing.

DISCUSSION

Before discussing the distribution and habitat of *Rana esculenta* complex in the Netherlands, attention is called to the other results of this study.

Most puzzling, although at this moment less important, is the discovery of albumin pattern BC which corresponds with albumin pattern Ar_1/Ar_2 found by TUNNER (1973) in Poland. It also occurs in the South of France and belongs to *ridibunda* (WIJNANDS, in preparation). Further discussion of its presence in the Netherlands is meaningful only after more detailed investigations have been made of the plasma albumins of green frogs from the surrounding countries.

The often large discrepancy between the serological and biometrical classification of frogs from various collecting stations (see Table II) cannot be completely explained at this moment; the sample sizes are too small to permit a detailed statistical analysis. However, if one adds up the numbers of frogs with albumin pattern B that are classified correctly and those that are misclassified from populations in which

albumin A and AB have not been found and compares these sums with those from mixed populations, a significant difference appears to exist: the relative number of misclassified frogs is higher in mixed populations (see Table III). In any case, this does not conflict with the suggestion (WIJNANDS, in preparation) that sympatry of all three forms allows gene flow and consequently a decrease of some of the differences between *lessonae* and *ridibunda*. Albumin BC has been left out of account in these considerations as it has only been found together with the other *ridibunda* pattern, B. That within albumin class AB no difference was found between the classification results in pure and mixed populations agrees with *esculenta* being a hybrid (Table III). Albumin class A and BC could not be analysed in this way, since no pure samples were available.

TABLE III

Frequency distributions of frogs that were classified correctly (*i.e.* were allotted to the albumin class to which they belong, on the basis of their biometrical characteristics) and frogs that were misclassified (*i.e.* were allotted to another albumin class on the basis of the values of the measurements of at least one body side) in pure and mixed populations (leaving albumin class BC out of account), cast in 2×2 contingency tables. Differences are tested by Chi²-tests (level of significance = .05). Albumin class A and BC could not be analysed in this way.

albumin B	*pure B-populations*	*mixed populations*
classified correctly	64	7
misclassified	11	7
$Chi^2 = 7.07$; df = 1		
p = .007		
albumin AB	*pure AB-populations*	*mixed populations*
classified correctly	37	51
misclassified	34	28
$Chi^2 = 1.82$; df = 1		
p = .177		

Because of the above-mentioned discrepancy between the serological and biometrical classification it should be kept in mind that in the following discussion of the distribution and habitat of *Rana esculenta* complex in the Netherlands the author does not pretend to draw conclusions as to the quantitative aspects of the populations sampled. This restriction is needed even more because of the often small sample sizes and the differences in time of sampling at the various collecting stations.

In contrast to WIJNANDS & VAN GELDER (1976), in this paper a frog

is considered to be identified if it is allotted to the albumin class to which it belongs, by application of the classification functions to the corresponding body side only. The results of both studies are used in the following considerations.

One may conclude that *ridibunda* prefers an environment abounding in water, as it is mainly found in the northern half of the country (Fig. 2) which is by far the richest in all sorts of waters. The type and

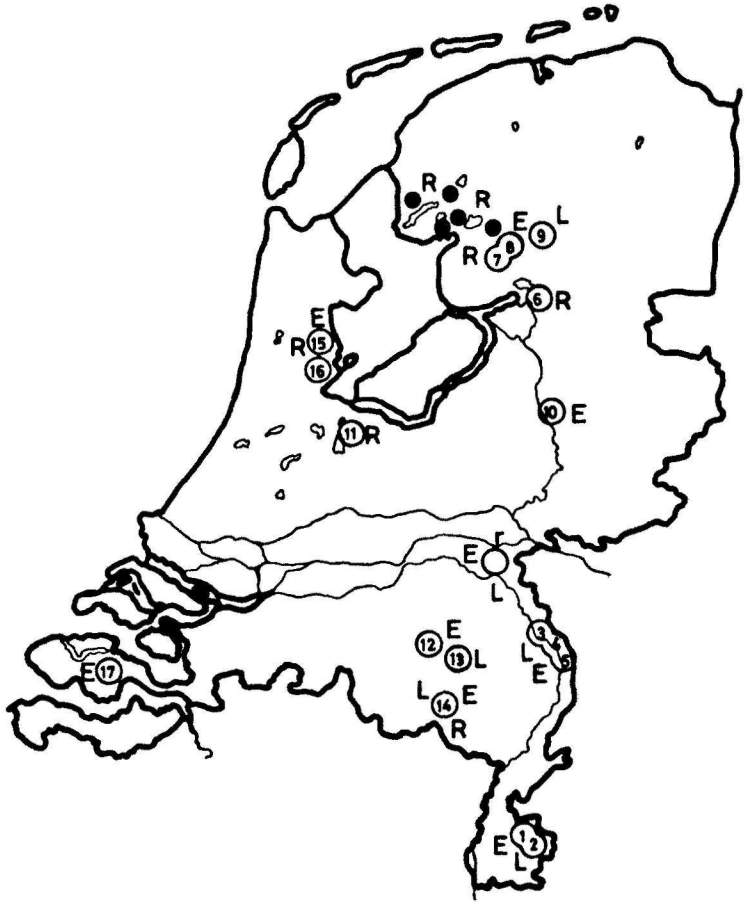

Fig. 2. Occurrence of *Rana esculenta* complex in various parts of the Netherlands. The encircled numbers refer to the collecting stations described in this paper. The black dots represent the collecting stations from a previous study (WIJNANDS & VAN GELDER, 1976) in the Province of Friesland. The area around Nijmegen, which is still investigated intensively, is indicated by an open circle without number. L = lessonae; E = esculenta; R = ridibunda; r = only incidental discoveries of ridibunda.

size of the water in which it occurs seems to be of minor importance. This does not fully agree with the data that are available in literature. TUNNER & DOBROWSKY (1976) assert that in those parts of Europe where the distribution areas of *lessonae* and *ridibunda* overlap, *ridibunda* only occurs in large waters like lakes and dead branches of rivers. For instance in Turkey, where *lessonae* does not occur, *ridibunda* lives in all sorts of waters according to these authors. To explain this, they start from the statement that hybridization of *lessonae* and *ridibunda* means loss of gene material for both species, in respect of their contribution to the next generation. The fact that *lessonae* and *ridibunda* seldom occur in the same water, would be an indication of the strong selection pressure which would act against interspecific matings and which would have led to a narrowing of ecologically adaptive properties falling within the range of the competing species (TUNNER & DOBROWSKI, 1976). Consequently, one would expect *ridibunda* to be more limited in its habitat selection in areas where *lessonae* also occurs. GÜNTHER (1974) discusses the distribution of green frogs in the German Democratic Republic and ascribes the diminished ecological plasticity of *ridibunda* in most parts of that country—in which *lessonae* and *esculenta* also occur—to the fact that it lives close to the border of its distribution area. The arguments of all three authors are plausible—although there is no evidence that they are correct—but they do not hold for the Netherlands: the present study shows that *lessonae* and *ridibunda* live here sympatrically (though seldom in the same water) but nevertheless *ridibunda* occurs in all sorts of waters, which conflicts with the above-mentioned explanation by TUNNER & DOBROWSKI (1976). Furthermore, *ridibunda* lives here, as in the German Democratic Republic, in the periphery of its distribution area, which extends roughly speaking from the Netherlands through Central and South-East Europe into South-West Asia (HOTZ, 1974). Therefore, GÜNTHER's (1974) argument does not apply to the Dutch situation either.

As to *lessonae* the results of this study agree with those of GÜNTHER (1974) and HEYM (1974). This form prefers smaller, often isolated waters. Speculating, one might say that the typical habitat of *lessonae* in the Netherlands consists of a mesotrophic fen in a wooded sandy area.

Esculenta can be found in all sorts of waters, which also agrees with the findings of GÜNTHER (1974) and HEYM (1974).

A rough picture of the distribution of the three forms is presented in Fig. 2.

In literature there are numerous reports of mixed populations of *esculenta* with either *lessonae* or *ridibunda*, whereas mixed populations of all three forms appear to be rare (*e.g.* HEYM, 1974). This agrees with

the results of this study. Only once *lessonae*, *esculenta* and *ridibunda* were found together in comparable numbers, namely in the river Kleine Dommel (collecting station 14). Especially the presence of *ridibunda* there is puzzling in view of its habitat and isolated position; the fact is that this collecting stattion does not lie in a watery area, comparable with the surroundings of the collecting stations of *ridibunda* in the northern half of the country.

Occasionally *ridibunda* has also been found in *esculenta-lessonae* populations near Nijmegen (unpublished data). The extremely low numbers there—4 specimens in all against about 500 *esculenta* and 400 *lessonae*—distributed over 2 years and 2 places make it plausible that these *ridibunda* are products of *esculenta* × *esculenta* matings. The fact is that breeding experiments in our laboratory, which are not finished yet, suggest that most *esculenta* × *esculenta* pairs (from Nijmegen) can produce large numbers of offspring which belong to *ridibunda* and *esculenta* and in any case can survive metamorphosis. Observations are still lacking as regards survival of *ridibunda* offspring from *esculenta* × *esculenta* pairs in the field.

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REFERENCES

BERGER, L., 1973. Systematics and hybridization in European green frogs of *Rana esculenta* complex.—J. Herpetol. **7**: 1–10.

GÜNTHER, R., 1973. Über die verwandtschaftlichen Beziehungen zwischen den europaischen Grunfroschen und den Bastardcharakter von *Rana esculenta* L. (Anura).—Zool. Anz. **190**: 250–285.

—, 1974. Neue Daten zur Verbreitung und Ökologie der Grunfrosche (Anura, Ranidae) in der DDR.—Mitt. Zool. Mus. Berlin **50**: 287–298.

—, & S. HAHNEL, 1976. Untersuchungen uber den Genfluss zwischen *Rana ridibunda* und *Rana lessonae* sowie die Rekombinationsrate bei der Bastardform *Rana 'esculenta'* (Anura, Ranidae).—Zool. Anz. **197**: 23–38.

HEYM, W.-D., 1974. Studien zur Verbreitung, Ökologie und Ethologie der Grünfrosche in der mittleren und nordlichen Niederlausitz.—Mitt. Zool. Mus. Berlin **50**: 263–285.

HOTZ, H., 1974. Ein Problem aus vielen Fragen—europaische Grunfrösche (*Rana esculenta*-Komplex) und ihre Verbreitung.—Natur und Museum **104**: 262–272.

TUNNER, H. G., 1973. Das Albumin und andere Bluteiweisse bei *Rana ridibunba*

PALLAS, *Rana lessonae* CAMERANO, *Rana esculenta* LINNÉ und deren Hybriden.—Z. zool. Syst. Evolut. forsch. **11**: 219–233.

—, & M.-Th. Dobrowsky, 1976. Zur morphologischen, serologischen und enzymologischen Differenzierung von *Rana lessonae* und der hybridogenetischen *Rana esculenta* aus dem Seewinkel und dem Neusiedlersee (Österreich, Burgenland). —Zool. Anz. **197**: 6–22.

Uzzell, Th. & L. Berger, 1975. Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae* and their hybridogenetic associate, *Rana esculenta*.—Proc. Acad. Nat. Sci. Philadelphia **127**: 13–24.

Van Gelder, J. J. & H. C. J. Oomen, 1970. Ecological observations on Amphibia in the Netherlands. I. *Rana arvalis* Nilsson: Reproduction, growth, migration and population fluctuations.—Neth. J. Zool. **20**: 238–252.

Vogel, P. & P. S. Chen, 1976. Genetic control of LDH isozymes in the *Rana esculenta* complex.—Experientia 32: 304–307.

Wijnands, H. E. J. & J. J. van Gelder, 1976. Biometrical and serological evidence for the occurrence of three phenotypes of green frogs (*Rana esculenta* complex) in the Netherlands.—Neth. J. Zool. **26**: 414–424.

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PARTIAL ECOLOGICAL ISOLATION OF RANA LESSONAE AND RANA ESCULENTA AS A MECHANISM FOR MAINTENANCE OF THE HYBRID FORM, RANA ESCULENTA (ANURA, RANIDAE)

by

H.E.J. WIJNANDS

With 1 figure and 6 tables

INTRODUCTION

There is hardly any doubt that in Europe three forms of green frogs occur: *lessonae*, *esculenta* and *ridibunda*. There also is sufficient evidence to justify the conclusion that *esculenta* originally results from hybridization of *lessonae* and *ridibunda* (BERGER 1973b, GÜNTHER 1973, TUNNER 1973). Things are less clear, however, if one tries to explain the way in which the *esculenta* form is maintained. Breeding experiments carried out by BERGER (1967, 1968a, 1970, 1976) and BLANKENHORN et al. (1971) showed that production of *esculenta* can almost solely take place through crosses of *lessonae* and *ridibunda* or backcrosses of *esculenta* and *lessonae*. TUNNER (1973, 1974) explained their results by assuming that within the hybrid-form the *lessonae*-genome is discarded during meiosis, a process which is known as hybridogenesis. Although this hypothesis has not yet been confirmed by cytological data, it is supported by the results of a study of LDH isozymes by VOGEL and CHEN (1976).

In contrast with BERGER (1967, 1968a, 1970, 1976) and BLANKENHORN et al. (1971), GÜNTHER (1973) found that *esculenta* is able to reproduce successfully even in complete absence of the other forms. As BERGER studied green frogs in Poland, BLANKENHORN et al. in Switzerland and GÜNTHER in the GDR, one is apt to ascribe the discrepancy between their findings to differences between the frogs they studied. This makes the whole problem even more complex than it is anyway.

In view of the discrepancy mentioned, the author held it necessary to make several small-scale breeding experiments of green frogs in the Nether-

lands, to be able to rate the results of his field studies at their true value. This paper presents the results of these investigations and especially discusses ecological aspects of the maintenance of *lessonae* and, consequently, of *esculenta*.

MATERIAL AND METHODS

Study area

The population studied lives close to the city of Nijmegen (5°51′ E. Long., 51°50′ N. Lat.), mainly in two State Reserves called "Overasseltse en Hatertse Vennen" and "Wijchens Ven". The former lies within a ridge of drift sand, measures about 285 hectares and consists of pine-forests (165 hectares), moorland (45 hectares), pastures and farming-land (45 hectares) and about 20 fens (30 hectares). Because of the presence of an impermeable layer of loam under the drift sand the fens can only be fed by rainwater, so that they originally were all oligotrophic. Some of the fens, however, are guanotrophicated by a colony of blackheaded gulls (*Larus ridibundus* Linnaeus) and some others are more or less enthrophicated as a result of agrarian activities. Several species of Amphibia occur in all the fens, but they are most numerous and can only reproduce successfully in the enriched ones. For a detailed description of this area the reader is referred to STRIJBOSCH (1976). The "Wijchens Ven" is an old branch of a river, lying close to the "Overasseltse en Hatertse Vennen". It consists of two parts of about 800 and 1300 metres in length and about 100 metres in width, partially separated by a dam. This eutrophic lake is almost totally surrounded by pastures. See figure 1 for a map of the area.

The wider surroundings of both reserves mainly consist of pastures and farming-land, with small woods, ponds, ditches, an occasional farm and, of course, the city of Nijmegen and some villages.

Population studies

As both the study area and the number of frogs living there was too large to intensively study the population as a whole, several pilot studies were made to select those fens which seemed most important for the frogs. Thereafter the fens selected ("Eendenven", "Ketelven", "Meeuwenven", "Roelofsven", "Kersjesven" and "Wijchens Ven"; see Figure 1) were investigated more systematically and intensively. Sampling took place by catching frogs by hand, with the aid of a dipnet or a fishing-rod, baited with a fishing-fly or simply a small petal. Only adult frogs (generally larger than 40 mms) were caught, both

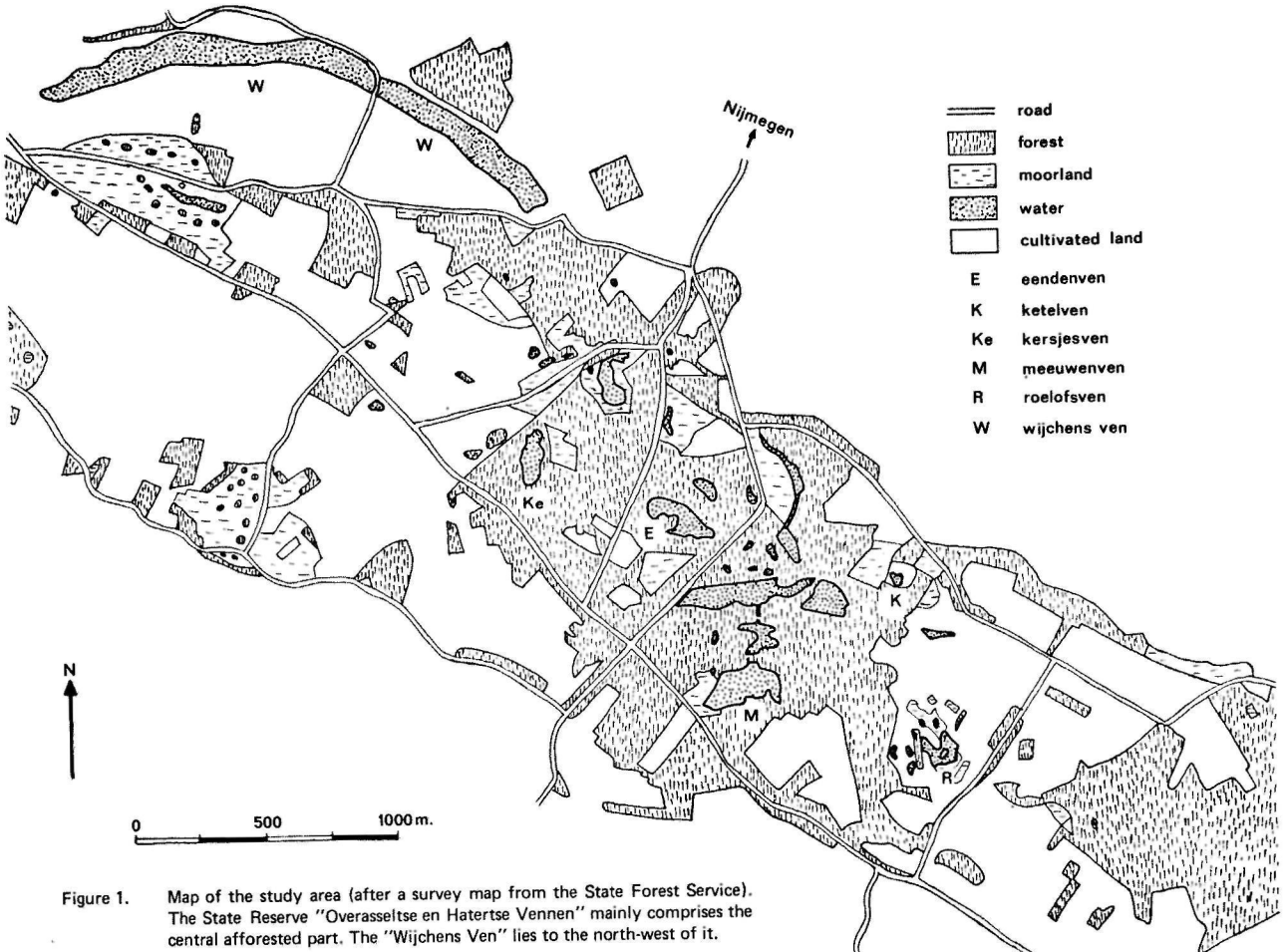

Figure 1. Map of the study area (after a survey map from the State Forest Service). The State Reserve "Overasseltse en Hatertse Vennen" mainly comprises the central afforested part. The "Wijchens Ven" lies to the north-west of it.

by day and by night, somewhat dependent on the weather conditions. They were measured and sometimes blood-sampled for identification (see below). They were released as soon as possible where they had been caught, after having been marked by means of toe-clipping. If too many frogs had been caught to be dealt with on the day of catching, they were kept at the most for a few days under proper conditions in the laboratory or in the field.

Breeding experiments

For breeding purposes ampletic pairs and single frogs that appeared willing to mate were caught and kept in pairs in the laboratory (or, in 1975 only, in a cage of wire netting placed in the "Roelofsven") until the eggs were deposited. Only those frogs were used for breeding, which by all criteria were identified as belonging to a certain form (see below). After the eggs had been laid, the parents were released where they had been caught. The number of eggs was determined and the eggs were kept in aerated tapwater in plastic tanks, lying on flywire 5-10 cms below water-level. After about two weeks, when the larvae had reached the stage in which they began to eat (stage 25; WITCHI 1956), the number of survivors was estimated and a known number of them were reared further (of each pair separately). In 1974 of each pair 200 larvae were reared in artificial ponds of about 250 litres, lying outdoors. In 1975 and 1977 125 and 100 larvae, respectively, were reared in plastic tanks (50 x 35 x 25 cms, containing 30 litres of tapwater) in a greenhouse, in which the temperature varied between 15° and 25°C (except in 1975, when it occasionally reached 35°C). Redundant tadpoles were set free where their parents had been caught. The larvae were fed nettle-agar (3% nettle-powder, 1% agar) that was squeezed into pulp and spread throughout the basins. The larvae were overfed a little bit. The water was changed twice a week and at the same time the larvae were counted, any food left was removed and new food supplied. As the tadpoles grew up, those that were kept in tanks were distributed over more tanks to reduce any influences of competitive interactions. Tadpoles that were kept in ponds were not distributed as the ponds seemed large enough to successfully rear them. Only in the tanks the water was aerated by air-pumps.

Metamorphosing tadpoles were put into slanting tanks (in the greenhouse) containing only enough water to cover half of the bottom, so that they could easily reach the shore. The juvenile frogs were kept in tanks, the bottom of which was covered with humid sand, declining to one end for a small puddle to develop. Some stones and plants were put in for shelter. The froglets were fed fruitflies, house-flies and small crickets.

Identification of the different forms

In 1976 WIJNANDS and VAN GELDER presented classification functions - that were obtained by means of discriminant analysis - by which almost 90% of the frogs studied could be classified correctly as either *lessonae*, *esculenta* or *ridibunda*. Initially, these functions were planned to be used for identification in the study that is reported upon here. In the course of the investigations, however, *ridibunda* appeared to be extremely rare in the study area. It therefore seemed more exact to use classification functions from a discriminiant analysis involving only two classes instead of three. These functions are:

$f_l = 26.36 - 1.73\ I_1 - 0.77\ I_3$, for the left side of the body and

$f_r = 27.15 - 1.78\ I_1 - 0.80\ I_3$, for the right side.

(I_1 = $Index_1$ = tibia length/length of callus internus; I_3 = $Index_3$ = tibia length/height of callus internus). Frogs that obtained positive scores for both functions were called *lessonae*, and frogs with negative scores *esculenta* (frogs of which the signs of both scores differed were considered not to be identified and were excluded from further studies; their number amounted to less than 5% of all of the frogs caught).

The identification of most adult frogs was exclusively based on their scores for the above classification functions. However, if reasonable doubt existed that any frog might belong to *ridibunda*, identification was based on the classification functions (with I_1 and I_3) for three classes and the plasma albumin pattern as well (see WIJNANDS and VAN GELDER 1976). The same was done for all frogs that were used for the breeding experiments.

Identification of tadpoles and juveniles rested solely on their plasma albumin pattern, as this was considered to be their only reliable characteristic. To obtain blood the animals were anaesthetized in a 0.05% solution of MS 222 (Sandoz, Basel) and then heart-punctured using a pointed glass capillary of 0.75 mm inner diameter. Further handling of the samples and the electrophoresis has already been described for frog-blood (WIJNANDS and VAN GELDER 1976). The only difference was that now about 4 µl of blood had to be used instead of 1 µl.

Breeding experiments

In total, eggs were obtained from 27 pairs: 20 *esculenta* x *esculenta*, 3 *lessonae* x *esculenta* (♂ x ♀) and 4 *lessonae* x *lessonae*. Some quantitative aspects of their reproductive capacity are summarized in Table I, whereas Table II presents the numbers of *lessonae*, *esculenta* and *ridibunda* in a sample of their offspring. Table II also shows data concerning offspring that were collected in the field as eggs or larvae, so that their parents were unknown. This applies to the sample called "Wijchens Ven" and to the sample from the "Berendonck" (a large new pond lying some two hundred metres north of the "Wijchens Ven"). The green frog population at the latter locality is only poorly known at the moment. *Esculenta* probably largely outnumbers *lessonae*, while *ridibunda* seems to be totally absent.

The number of hatchlings was estimated in 1977 only. Their average number amounted to 75% of the eggs from known *esculenta* x *esculenta*-pairs. Of the eggs that were collected in the "Berendonck" 90% hatched. Abnormal differences between egg-sizes were not observed, except in one case: the eggs from *esculenta* x *esculenta*-pair 7 in 1975 (see Table VI) were very variable in size, the largest eggs having a diameter of more than twice that of the smallest ones.

In general, all juveniles resulting from *esculenta* x *esculenta*-matings were feeble creatures, clumsy in jumping and poor eaters. Other obvious anomalies of larvae and especially juveniles of such pairs were: inability to bend the knee-joint of one or both hindlegs (about half of the offspring of one pair in 1975 and one in 1977), swollen body and poorly developed internal organs (all of the offspring that died before stage 26, WITCHI 1956) and swollen lymph sacs (about one third of the offspring of one pair in 1977). Sporadically, the latter anomaly occurred among offspring of one *lessonae* x *lessonae*-pair and among larvae from eggs collected in the "Berendonck" too. Once in a while, it also occurs among offspring from *Xenopus*, which is bred for other purposes in another laboratory at Nijmegen. Thus, the appearance of this anomaly is not typical of *esculenta* x *esculenta*-offspring, but, because of its massed occurrence among these, it just might reflect their lower viability.

In view of all this, it is not surprising that mortality of the juveniles was very high. Only ten froglets from one pair (*esculenta* x *esculenta*, 1974) lived for slightly more than a year, all of the remaining offspring from known *esculenta* x *esculenta*-pairs died within 5 months after metamorphosis, most of them even within two months. In 1975 this could have partly been due to the fact that the temperature in the greenhouse could not be kept within appro-

Table I. Quantitative aspects of the results of the breeding experiments. E = *esculenta*; L = *lessonae*; n = number.

	1974 E x E	1975 E x E	L♂ x E♀	L x L	1977 E x E
n of pairs	5	8	3	4	7
mean n of eggs per egg-mass	unknown	2180	1670	800	1160
no development (n of egg-masses)	1	1	0	0	0
all larvae dead before stage 26* (n of egg-masses)	1	0	0	0	4
remaining, developing "egg-masses"	3	7	3	4	3
mean % of metamorphosing larvae**	75	80	80	85	95

* According to WITCHI (1956).

** Relative to the number of larvae reared from stage 25.

priate limits, as mortality of the offspring from *lessonae* x *lessonae* and *lessonae* x *esculenta* was high too. In 1977, however, among the juveniles resulting from eggs and larvae that were collected in the "Berendonck" and the "Wijchens Ven", respectively, there were several specimens of *lessonae* (see Table II), which could be recognized by their bright green colour. These did very well, in contrast with the brownish *ridibunda* with which they grew up. Furthermore, juveniles originating from tadpoles that were caught in an area in which *ridibunda* predominates ("Ankeveen", see WIJNANDS 1977) grew extremely well (these froglets probably all belong to *ridibunda*, judging from their brownish colour and body proportions; up to now, however, an objective

Table II. Numbers of the different forms in samples of offspring (= metamorphosing tadpoles and juveniles) from known parents and from eggs and larvae collected in the field. L = *lessonae*; E = *esculenta*; R = *ridibunda*; W.V. = Wijchens Ven; Ber. = Berendonck, a large pond close to the Wijchens Ven.

Origin	Offspring			Origin	Offspring			Origin	Offspring		
	L	E	R		L	E	R		L	E	R
1974				1975				1977			
E x E 1	0	0	34	L♂ x E♀ 1	0	17	0	E x E 1	0	0	18
2	0	0	18	2	0	14	0	2	0	0	20
3	0	0	22	3	0	21	0	3	0	1	19
1975											
E x E 1	0	0	19	L x L 1	21	0	0	W.V.	4	0	17
2	0	0	23	2	30	0	0	Ber.	15	3	32
3	0	0	23	3	24	0	0				
4	0	2	15	4	23	0	0				
5	0	2	19								
6	0	3	16								
7	0	9	4								

identification involving plasma electrophoresis has not been made, as they have been destined for further experiments). It seems, therefore, that the low viability of *esculenta* x *esculenta*-offspring is mainly determined by their descent.

Field studies

For the sake of clearness, the results of the field studies are presented in three parts, as they were obtained in three experiments that were based on the outcome of the pilot studies.

1) Occurrence of *lessonae* and *esculenta* in four fens selected.

In 1975 and 1977 four fens were investigated in respect of the relative numbers of *lessonae* and *esculenta* living there (*ridibunda* was only found in the "Wijchens Ven", as is shown below). Two of these fens, "Eendenven" and "Meeuwenven", belong to the guanotrophicated ones, the other two, "Ketelven" and "Roelofsven", are eutrophicated (STRIJBOSCH 1976). In 1975 samples were taken by day during the spawning season, from early May until half June. As attention was especially directed at the frogs taking part in reproduction, mainly ampletic pairs were caught, place and time of catching being strongly dependent on the breeding activity of the frogs. In 1977 the fens were sampled simultaneously during four successive nights in August, clearly after the breeding season. Table III presents the numbers of *lessonae* and *esculenta* caught at the relative occasions. In both years the relative frequencies of *lessonae* and *esculenta* were significantly different over the four fens. The results of 2x2-tests are shown in Table IV. In Table III and IV males and females are taken together, as preliminary tests showed that their relative numbers were fairly equal in all fens (separately for *lessonae* and *esculenta*). This, of course, is only relevant for 1977 as in 1975 mainly ampletic pairs were caught.

2) Change of the relative numbers of active *lessonae* and *esculenta* in the course of the year.

The results of the pilot studies suggested a decline of the ratio of the numbers of active (i.e. catchable in the fen) *lessonae* and *esculenta* in the course of the year. To verify this, in 1976 a study was started in another guanotrophicated fen (STRIJBOSCH 1976), called "Kersjesven" (see Fig. 1), where both forms seemed to occur in comparable numbers. It was intended to take samples of the subpopulation living in this fen as often as possible (at least once a week), starting in April when the number of active frogs begins to rise, right before their breeding period and continuing until September when hibernation is at hand. Unfortunately, the "Kersjesven" completely dried up already in July, as a result of the extremely dry and hot summer, so that the investigation ended too soon. To prevent this from happening again, attention was shifted to the frogs in the "Meeuwenven" and the investigation outlined above was started anew in 1977.

Table III. Numbers of *lessonae* and *esculenta* caught in four fens selected of the "Overasseltse en Hatertse Vennen". Level of significance = .05.

1975 (May-June)	Eendenven	Ketelven	Meeuwenven	Roelofsven	Total
lessonae	5	50	19	134	208
esculenta	11	8	8	8	35
total	16	58	27	142	243

$Chi^2 = 52.28$; $df = 3$; $p < .001$.

1977 (August)					
lessonae	53	61	36	88	238
esculenta	45	22	50	16	133
total	98	83	86	104	371

$Chi^2 = 44.99$; $df = 3$; $p < .001$.

In view of a meaningful statistical analysis the numbers of frogs caught daily had to be added to a total of longer periods. As the "Kersjesven" was small and accessible enough to sample it as a whole within a few days, the adding was carried out within periods of 20 days. The "Meeuwenven" could not be sampled within a few days. Therefore, in this case the adding took place within the periods needed to complete one "sampling-round", varying from 7 to 30 days. Table V presents the numbers of individual frogs caught and the results of tests for trend (VAN EEDEN 1955). According to these tests there is a significant negative trend in the relative numbers of *lessonae* caught in the successive periods, except for the males of "Kersjesven" 1976. Unfortunately, the numbers of ampletic pairs caught were too low to allow a separate analysis of any changes of the frequencies of the different combinations that might occur in the course of the breeding season.

Table IV. Results of 2x2-Chi^2-tests, applied to the numbers of *lessonae* and *esculenta* caught in four fens selected (see Table III). Level of significance for each separate 2x2-table is .05/6 = .008, in order to obtain a simultaneous level of significance of less than .05 over all six 2x2 comparisons for each year; + means: significant difference between the relative frequencies of *lessonae* and *esculenta* for the fens compared; – means: no such difference; df = 1.

			1975			1977		
			Chi^2	p		Chi^2	p	
Eendenven	x	Ketelven	17.07	< .001	+	6.45	< .02	–
Eendenven	x	Meeuwenven	4.75	< .05	–	2.27	> .10	–
Eendenven	x	Roelofsven	48.35	< .001	+	20.89	< .001	+
Ketelven	x	Meeuwenven	2.08	> .10	–	16.01	< .001	+
Ketelven	x	Roelofsven	2.70	> .10	–	2.87	> .10	–
Meeuwenven	x	Roelofsven	12.57	< .001	+	36.09	< .001	+

3) The *esculenta-lessonae-ridibunda*-population of the "Wijchens Ven".

The green frogs inhabiting the "Wijchens Ven" excited interest immediately after the pilot studies had started in 1974. The first impression was that this subpopulation consisted almost exclusively of *esculenta*. Although this impression did not cover the facts, yet more intensive studies showed that *esculenta* largely outnumbered *lessonae*. *Ridibunda* appeared to occur here too; already in 1975 2 females of this form were caught. Unfortunately, the "Wijchens Ven" is far too large and inaccessible to allow similar investigations as in the "Kersjesven" or "Meeuwenven". Therefore, Table VI shows only the total numbers of individual frogs, that were caught between April and September of 1976 and of 1977. Chi^2-tests revealed that of *esculenta* significantly more females than males were caught, both in 1976 and in 1977 (Chi^2 = 16.99 and 163.99, respectively, df = 1, $p < .001$). For *lessonae* no such differences were found (1976: Chi^2 = 1.94; 1977: Chi^2 = 1.44; df = 1, $p > .10$). In both years significantly more *esculenta* were caught than *lessonae*, males as well as females.

Table V. Change of the relative numbers of active *lessonae* and *esculenta* in the course of the year. Level of significance = .05.

Kersjesven 1976	Period			
	20/4– 9/5	10/5– 29/5	30/5– 18/6	19/6– 8/7
♂				
lessonae	43	73	28	33
esculenta	2	11	3	6
♀				
lessonae	23	23	13	28
esculenta	2	2	10	9

Test for trend (VAN EEDEN 1955): ♂ $u = -1.357$, $p > .10$

(total = breeding period) ♀ $u = -2.495$, $p < .02$

Meeuwenven 1977	Period						
	2/5– 18/5	18/5– 8/6	8/6– 29/6	29/6– 29/7	29/7– 10/8	10/8– 19/8	26/8– 1/9
♂							
lessonae	43	55	39	75	44	11	7
esculenta	11	17	19	50	28	21	9
♀							
lessonae	21	49	50	92	60	25	26
esculenta	5	23	48	106	60	29	18

Test for trend (VAN EEDEN 1955): ♂ $u = -4.240$, $p < .001$

(total) ♀ $u = -2.623$, $p < .01$

over the first four periods: ♂ $u = -2.818$, $p < .005$

(= breeding period) ♀ $u = -3.727$, $p < .001$

Table VI. Numbers of *lessonae*, *esculenta* and *ridibunda* caught in the "Wijchens Ven" in 1976 and 1977.

1976	lessonae	esculenta	ridibunda	Total
♂	21	41	0	62
♀	12	89	1	102
total	33	130	1	164
1976				
♂	29	80	0	109
♀	40	345	9	394
total	69	425	9	503

DISCUSSION

Comparison of the results of breeding experiments of BERGER (1967, 1968a, 1970, 1971a + b, 1973a + b, 1976), BLANKENHORN et al. (1971), GÜNTHER (1973) and those obtained in this study shows that the reproductive ability of *esculenta* is rather variable. BERGER (1967-1976) and BLANKENHORN et al. (1971) found that crosses of *esculenta* and *lessonae* exclusively yield *esculenta*, while *esculenta* x *esculenta*-matings seldom or never produce offspring that complete metamorphosis and if so only *ridibunda*. GÜNTHER (1973) confirmed their results as to crosses of *lessonae* and *esculenta* but found that *esculenta* by itself produced *esculenta*, *ridibunda* or even *lessonae*-like offspring, all of which were more or less vital and fertile. In the present study the results of the few crosses of *lessonae* and *esculenta* made, agreed with those of the authors mentioned above. *Esculenta*-pairs mainly produced *ridibunda* and occasionally *esculenta*, most of which survived metamorphosis. However, on the basis of the low vitality of this offspring in the laboratory and because of the fact that *ridibunda* is extremely rare in the area studied, it is doubted that they contribute much to reproduction in the field.

It will be clear that the variability outlined above provides a serious

drawback for the progress of the investigations of the *Rana esculenta* complex. Yet, it seems justified to conclude that at least in some parts of Europe, production of *esculenta* in the field mainly depends on the presence of *lessonae*, as *lessonae* and *ridibunda* seldom occur in the same habitat. Since backcrosses of *lessonae* and *esculenta* exclusively yield *esculenta*, the question arises how *lessonae* is protected from "over-exploitation" by *esculenta*. The following considerations will show that this could be accomplished by the partial ecological isolation of the two forms, that was observed in this study.

Theoretically speaking, in a mixed population of *lessonae* and *esculenta* the relative probability of backcrosses (*esculenta* x *lessonae* and vice versa) is twice that of *lessonae* x *lessonae*-matings. Furthermore, let us assume for the moment, that all three productive combinations (LxL, LxE, ExL) produce equal numbers of offspring of equal chances of survival and reproduction. In that cases, the number of *esculenta* relative to *lessonae* would rapidly increase, which could eventually lead to extinction of *lessonae* and, consequently, of *esculenta*, at least in local populations. Of course, immigration could delay such a dramatic end, but nevertheless one would expect large fluctuations of population densities to occur which, from an evolutionary point of view, do not seem very favourable for survival.

The partial spatial and temporal isolation of *lessonae* and *esculenta* that was found in this study could be one of the means of securing a more stable situation. Partial spatial isolation could result in the existence of local subpopulations of *lessonae*, that are relatively "undisturbed" by *esculenta* (in this study in "Ketelven" and "Roelofsven"); by means of dispersal neighbouring mixed subpopulations in which *esculenta* is more numerous (here in "Eendenven" and "Meeuwenven") could be supplied with highly necessary *lessonae*. Partial temporal isolation, *esculenta* becoming active later than *lessonae*, could reduce the probability of backcrosses, thus directly achieving the required effect.

One might argue that the above picture strains reality at several points. In the first place, *esculenta* ♂ x *lessonae* ♀-matings are less likely to occur in the field than other ones, since in ampletic pairs the male is generally smaller than the female. Yet, such matings frequently occur according to our observations and there is no reason to believe that they are much less numerous than can be expected on the basis of chance. Thus, maybe the relative probability of backcrosses is not as much as twice the probability of *lessonae* x *lessonae*-matings, but it surely exceeds it clearly. Secondly, many *esculenta*-frogs, especially males, suffer from reduced fertility (GÜNTHER 1973). This alone, indeed, could be sufficient to keep their number at a rather constant level, obviating the need for other regulatory mechanisms. However, BERGER (1967) states that all three forms are highly fertile. Thus, the regulatory effect of the fertili-

ty of *esculenta* probably is not equally important in all populations. Furthermore, it will be counteracted by the fact that *esculenta*-females will generally produce more eggs, simply because they are bigger than *lessonae*-females.

There is another factor that might play an imortant role, namely the chance of survival of *esculenta* relative to that of *lessonae*. Numerous data are available about vitality of green frogs bred in the laboratory, but field data are totally lacking in this respect. Although BERGER (1968b) and HEUSSER and BLANKENHORN (1973) made laboratory experiments to determine inhibitory interactions of green frog tadpoles belonging to different forms, it would go too far to apply their conclusions to natural populations. As regards juveniles and adults no such data are available at all.

Obviously, the hypothesis presented above needs support of many observations, some of which are very hard to make. Anyhow, there is evidence that partial ecological isolation of *lessonae* and *esculenta* exists, but the magnitude of its regulatory effect on population structure and survival of both forms has to be further investigated.

BLANKENHORN (1974) made ethological and ecological observations of *lessonae-esculenta*-populations in Switzerland. He suggests that the variability of the relative numbers of *lessonae* and *esculenta* is a mean in achieving adaptation to different habitats. It is not clear, however, in which way this adaptation comes about. In fact, it seems impossible that *esculenta* can make a positive contribution to survival of the population. It is only present thanks to *lessonae*.

As regards the difference between the habitat preferred by *lessonae* and *esculenta* we can only suppose that the size of the water plays an important role, *lessonae* predominating in small waters, *esculenta* in larger ones. It is true that, within the area of the "Overasseltse en Hatertse Vennen", *esculenta* avoids the fens that are eutrophicated by agrarian activaties. However, if this would constitute the main difference with *lessonae*, *esculenta* would have to avoid the "Wijchens Ven" too, as this lake undoubtedly is eutrophicated by the surrounding pastures. The only rough resemblance between the waters preferred by *esculenta* seems to lie in their relative expanse, but further examinations are needed to clarify this point.

In literature there are several reports of abnormal sex-ratios among offspring of green frogs bred in the laboratory (BERGER 1971a + b, GÜNTHER 1973), but field observations are scarce in this respect (GÜNTHER 1968, TUNNER 1974). In any case, our data from the "Wijchens Ven", where *esculenta*-females largely outnumber males, resemble TUNNER's findings (1974) in the *esculenta-lessonae*-population at the "Neusiedlersee" in Austria.

The variability of egg sizes and the abnormalities in larval development encountered in this study are not surprising, as they have been described

before (BERGER 1967, 1968a, GÜNTHER 1970). On the basis of BERGER's findings (1976) one might expect the (relatively numerous) *esculenta*-offspring of the pair that produced eggs of different sizes to be triploid, but this was not examined.

Finally, one may ask whether *ridibunda* plays an important role in maintaining the *esculenta*-form in the field. If backcrosses of *esculenta* and *ridibunda* really yield *ridibunda* only (BERGER 1976), the existence of mixed populations of both forms has to depend on reproduction of *esculenta* by itself. This seems impossible on the basis of BERGER's results. Furthermore, it seems unlikely that *esculenta* from the area studied here is able to do so. This would mean that *esculenta* from Nijmegen possesses other characteristics than those in the northern half of the Netherlands, where *ridibunda* is very abundant and also lives together with *esculenta* (WIJNANDS 1977). Perhaps in this area the situation resembles that encountered by GÜNTHER (1968, 1973) in some parts of the GDR. Up to now, this has not been examined, for practical reasons mainly. The fact is that, unlike the area studied here, the environment in which *ridibunda* lives (in the Netherlands) is not surveyable from the viewpoint of the population ecologist. Perhaps in other parts of Europe the situation is more favourable in this respect.

SUMMARY

During several years a population of green frogs (*Rana esculenta* complex) was studied in an area (about 400 hectares) consisting of afforested sanddunes, moorland, farming land, pastures and several waters of very different sizes and degrees of eutrophication. The main object of the study was to get information about the way in which the hybrid form (*esculenta*) is maintained. For this purpose, breeding experiments were made as well as studies of the population structure - expressed as the relative numbers of active frogs of the different forms - in different waters and in the course of the year.

Ridibunda appeared to be extremely rare in the area studied, whereas *lessonae* and *esculenta* were found to occur in comparable numbers. However, a significant difference existed between the relative numbers of *lessonae* and *esculenta*, living in different waters within the study area: in smaller pools *lessonae* outnumbered *esculenta*, while in larger ones *esculenta* was at least equinumerous to *lessonae*.

There appeared to be a significant trend of the relative numbers of active *lessonae* and *esculenta* in the course of the year: in early May, at the beginning of the breeding season, *lessonae* outnumbered *esculenta*; thereafter the relative

number of *esculenta* increased to match that of *lessonae* in about August, after the breeding season.

The main result of the breeding experiments was that *esculenta* x *esculenta*-pairs produced *ridibunda* and a few *esculenta*. However, in view of the low vitality of this offspring (in the laboratory) they are not considered to be able to survive and reproduce successfully in the field.

All these results and literature data are discussed in respect of the way in which the *esculenta*-form is able to survive. It is suggested that partial ecological isolation of *lessonae* and *esculenta* is one of the means in achieving this.

LITERATURE

BERGER, L. (1967): Embryonal and larval development of F_1 generation of green frogs different combinations. - Acta Zool. Crac. 12, 123-160.

BERGER, L. (1968a): Morphology of the F_1 generation of various crosses within *Rana esculenta* complex. - Acta Zool. Crac. 13, 301-324.

BERGER, L. (1968b): The effect of inhibitory agents in the development of greenfrog tadpoles. - Zool. Polonae 18, 381-390.

BERGER, L. (1970): Some characteristics of the crosses within *Rana esculenta* complex in postlarval development. - Ann. Zool. 27, 373-416.

BERGER, L. (1971a): Inheritance of sex and phenotype in F_1 and F_2 crosses within *Rana esculenta* complex. - Genetica Polonica 21, 517-521.

BERGER, L. (1971b): Viability, sex and morphology of F_2 generation within forms of *Rana esculenta* complex. - Zool. Poloniae 21, 345-393.

BERGER, L. (1973a): Some characteristics of backcrosses within forms of *Rana esculenta* complex. - Genetica Polonica 14, 413-429.

BERGER, L. (1973b): Systematics and hybridization in European green frogs of *Rana esculenta* complex. - J. Herpetol. 7, 1-10.

BERGER, L. (1976): Hybrids of B_2 generations of European water frogs (*Rana esculenta* complex). - Ann. Zool. 33, 201-214.

BLANKENHORN, H.J. (1974): Soziale Organisation einer Mischpopulation von *Rana lessonae* Camerano und *Rana esculenta* Linnaeus. - Dissertation, Zürich.

BLANKENHORN, H.J., H. HEUSSER and P. VOGEL (1971): Drei Phänotypen von Grünfröschen aus dem *Rana esculenta*-Komplex in der Schweiz. - Rev. Suisse Zool. 78, 1243-1247.

EEDEN, C. VAN (1955): Een toets tegen verloop voor een aantal kansen. - Statistica Neerlandica 9, 131-159.

GÜNTHER, R. (1968): Morphologische und ökologische Untersuchungen zur Unterscheidung von *Rana esculenta* L. und *Rana ridibunda* Pall. - Zool. Jb. Syst. 95, 229-264.

GÜNTHER, R. (1970): Der Karyotyp von *Rana ridibunda* Pall. und das Vorkommen von Triploidie bei *Rana esculenta* L. (Anura, Amphibia). - Biol. Zentralbl. 89, 327-342.

GUNTHER, R. (1973) Uber die verwandtschaftlichen Beziehungen zwischen den europaischen Grunfroschen und den Bastardcharakter von *Rana esculenta* L. (Anura) - Zool. Anz 190, 250-285

HEUSSER, H. and H.J. BLANKENHORN (1973) Crowding-Experimente mit Kaulquappen aus homo- und heterotypischen Kreuzungen der Phanotypen *esculenta, lessonae* und *ridibunda* (*Rana esculenta*-Komplex, Anura, Amphibia). - Rev. Suisse Zool 80, 543-569.

STRIJBOSCH, H. (1976) Een vergelijkend syntaxonomische en synoecologische studie in de Overasseltse en Hatertse Vennen bij Nijmegen - Dissertation, Nijmegen.

TUNNER, H.G. (1973) Das Albumin und andere Blutweisse bei *Rana ridibunda* Pallas, *Rana lessonae* Camerano, *Rana esculenta* Linné und deren Hybriden. - Z. zool. Syst. Evolut forsch 11, 219-233.

TUNNER, H.G. (1974) Die klonale Struktur einer Wasserfroschpopulation - Z. zool Syst. Evolut forsch 12, 309-314.

VOGEL, P and P.S. CHEN (1976) Genetic control of LDH isozymes in the *Rana esculenta* complex. - Experientia 32, 304-307.

WIJNANDS, H.E.J (1977) Distribution and habitat of *Rana esculenta* complex in the Netherlands. - Neth, J. Zool 27, 277-286

WIJNANDS, H E.J. and J.J VAN GELDER (1976) Biometrical and serological evidence for the occurrence of three phenotypes of green frogs (*Rana esculenta* complex) in the Netherlands. - Neth. J Zool 26, 414-424.

WITCHI, E. (1956) Development of Vertrebates Philadelphia.

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In het eerste hoofdstuk van dit proefschrift worden biometrische en serologische gegevens gepresenteerd, die tot de conclusie leiden dat in Nederland drie vormen van groene kikkers (*Rana esculenta* complex) voorkomen: *lessonae*, *esculenta* en *ridibunda*. De biometrische kenmerken, die het meest bruikbaar zijn om deze vormen van elkaar te onderscheiden, zijn de relatieve tibialengte en de relatieve lengte en hoogte van de callus internus. *Lessonae* heeft de kortste tibia en de grootste callus, *ridibunda* respectievelijk de langste en kleinste. *Esculenta* is ten aanzien van deze kenmerken, uiteraard, intermediair. *Lessonae* en *ridibunda* zijn goed van elkaar te onderscheiden. De kenmerken van *esculenta* overlappen die van de beide andere vormen echter aanzienlijk. Daarom werd discriminant-analyse gebruikt voor het vaststellen van duidelijke kriteria voor de identificatie. De indeling in klassen die hiervoor nodig was kwam tot stand op basis van verschillen in het plasma-albuminepatroon, dat bepaald werd met behulp van polyacrylamide-gel-electroforese. In het materiaal dat in dit stadium van het onderzoek werd bestudeerd bleken slechts drie albuminepatronen voor te komen, namelijk A (een relatief snelle band) bij *lessonae*, B (een langzamere band) bij *ridibunda* en AB (beide banden) bij *esculenta*.

In hoofdstuk II wordt, aan de hand van in Nederland en Frankrijk verzamelde gegevens, nader ingegaan op genoemde biometrische en serologische kenmerken. In Nederland blijkt een tweede albuminepatroon van *ridibunda* voor te komen (BC, waarbij de C-band langzamer is dan de B-band), zij het zeer zeldzaam*. Alle vier tot nu toe genoemde patronen (A, AB, B, BC) komen ook in Frankrijk voor. De beschikbare gegevens laten niet toe een bepaald albuminepatroon (B of BC) te koppelen aan een bepaalde subspecies van *ridibunda* (*Rana r. ridibunda* of *Rana r. perezi*). Tussen de onderzochte biometrische kenmerken van *ridibunda* met verschillend albuminepatroon blijken geen significante verschillen te bestaan. Dit is wel het geval tussen *ridibunda* (met albuminepatroon B) uit Frankrijk en Nederland: de Nederlandse *ridibunda* lijkt meer op *esculenta* dan de Franse. Aangezien in Nederland behalve *ridibunda* ook *lessonae* en *esculenta* volop voorkomen, terwijl het gebied dat in Frankrijk werd bemonsterd waarschijnlijk zuivere *ridibunda*-populaties herbergt, zou dit erop kunnen wijzen dat een zekere mate van introgressie tussen *lessonae* en *ridibunda* mogelijk is en dat bij *esculenta* dus geen volledige hybridogenese optreedt.

Biometrische gegevens uit Hoofdstuk III ondersteunen de suggestie dat bij *Rana esculenta* geen volledige hybridogenese optreedt (zie hoofdstuk II). In dit hoofdstuk wordt verder een globaal beeld gegeven van de verspreiding van de drie vormen in Nederland. Tevens wordt aangegeven in of bij welk type

water ze het meest worden aangetroffen. *Ridibunda* blijkt vooral voor te komen in waterrijke gebieden, zoals polders met veel sloten en in de buurt van groot open water. Het is dan ook niet verwonderlijk dat deze vorm zeer talrijk is in de noordelijke helft van het land en dat hij in het zuiden slechts sporadisch voorkomt. *Lessonae* kan in vrijwel het hele land worden aangetroffen. Hij leeft in en bij kleinere, soms zelfs tamelijk geïsoleerde wateren. *Esculenta* komt waarschijnlijk overal voor, in allerlei typen water. Hij leeft meestal samen met een van beide andere vormen, zelden met alle twee.

Hoofdstuk IV behandelt aspecten van het voortbestaan van *esculenta* op populatieniveau. Uit kweekexperimenten blijkt dat, althans in het hier bestudeerde gebied, *esculenta* vrijwel uitsluitend geproduceerd wordt door terugkruising met *lessonae*, met andere woorden *lessonae* moet behalve zichzelf ook *esculenta* in stand houden. Er moeten dus speciale mechanismen zijn om een voldoende produktie van *lessonae* te waarborgen, anders zullen beide vormen verdwijnen. Zo'n mechanisme zou kunnen zijn een gedeeltelijke tijdelijke en ruimtelijke isolatie van *esculenta* en *lessonae*, die in het terrein van onderzoek inderdaad blijkt te bestaan.

* Inmiddels is de C-band ook als afzonderlijk patroon aangetroffen, doch slechts bij één *ridibunda* in Nederland, in dezelfde populatie waaruit ook de exemplaren met het BC-patroon stammen (WIJNANDS, niet gepubliceerd). Bovendien blijken ook bij *lessonae* drie albuminepatronen voor te komen, namelijk het reeds bekende A, verder A' (met een iets grotere loopsnelheid dan A) en A'A (beide banden); bij *esculenta* is de A'-band ook gevonden, in het A' B-patroon (GÜNTHER & LÜBCKE 1979; WIJNANDS, niet gepubliceerd).

Hoe het *Rana esculenta* complex zich verder zal ontwikkelen zal vooral afhangen van de wijze waarop de *esculenta*-vorm in stand gehouden wordt. Hierbij zal het voorkomen van triploide *esculenta* (zie GÜNTHER 1970, 1975a en b; UZZELL & BERGER 1975; UZZELL, BERGER & GÜNTHER 1975) waarschijnlijk een grote rol spelen. Het lijkt erop, hoewel de beschikbare gegevens nog lang niet volledig zijn, dat er een verband bestaat tussen het aandeel van *esculenta* in de populatie en het percentage triploide *esculenta* dat in de populatie aanwezig is. In populaties waarin *esculenta* en een van zijn oudersoorten (veelal *lessonae*) ongeveer in gelijke aantallen voorkomen - populaties van alle drie vormen samen zijn zeer zeldzaam - is het percentage triploiden als regel laag (GÜNTHER 1975b). Zo'n situatie doet zich bijvoorbeeld voor in de populatie die in hoofdstuk IV van dit proefschrift werd besproken (WIJNANDS, niet gepubliceerd).

In dergelijke populaties is *esculenta* voor zijn voortplanting afhankelijk van terugkruising met de betreffende oudersoort (hybridogenese). Hoewel zo'n systeem op het eerste gezicht geen lang leven beschoren lijkt, is binnen het subfylum Vertebrata toch al een twintigtal min of meer vergelijkbare gevallen bekend, waarvan er geen binnen afzienbare tijd dreigt te verdwijnen (VRIJENHOEK et al. 1978). Het betreft hier "soorten" die uitsluitend uit vrouwelijke exemplaren bestaan en die zich voortplanten door middel van gynogenese, bijvoorbeeld sommige *Ambystoma* (Amphibia) (UZZELL 1963), of hybridogenese, bijvoorbeeld sommige *Poeciliopsis* (Pisces) (SCHULTZ 1969). Van enkele van deze gevallen is ook bekend dat er oecologische of ethologische mechanismen zijn om te voorkomen dat de hybride-vorm de oudersoort, waarvan hij voor zijn voortplanting afhankelijk is, verdringt en daarmee zichzelf uitroeit (WILBUR 1971; MOORE 1975).

De kans dat de hybriden een blijvend evolutionair succes behalen is natuurlijk groter als ze in staat zullen zijn zich onafhankelijk van hun oudersoorten voort te planten. Parthenogenese zou een manier kunnen zijn om dit te bewerkstelligen. Er zijn inderdaad enkele door hybridisatie ontstane vertebraten-soorten die zich op deze wijze voortplanten, onder andere hagedissen uit het geslacht *Lacerta* (UZZELL & DAREVSKY 1975). Er is momenteel echter geen reden om aan te nemen dat *Rana esculenta* zich parthenogenetisch voortplant, ook niet in de weinige zuivere populaties (zie GÜNTHER 1975b) die van deze vorm bekend zijn. Hoe deze populaties in stand worden gehouden is nog niet helemaal duidelijk, maar hierbij spelen triploiden, waarvan juist in deze populaties de hoogste percentages zijn gevonden (GÜNTHER 1975b), waarschijnlijk een grote rol (UZZELL et al. 1979). In ieder geval lijkt de kans op blijvend succes van *Rana esculenta*, bijvoorbeeld door het ontstaan van een

tetraploid die zich voortplant via normale meiotische processen (vgl. SCHULTZ 1969; WILBUR 1971), in deze populaties het grootst.

LITERATUUR

BERGER, L., 1973. Systematics and hybridization in European green frogs of *Rana esculenta* complex.- J. Herpetol. 7 1-10

GUNTHER, R , 1970. Der Karyotyp von *Rana ridibunda* Pall und das Vorkommen von Triploidie bei *Rana esculenta* L. (Anura, Amphibia).- Biol. Zentralbl. 89 327-342.

GUNTHER, R. 1973. Uber die verwandtschaftlichen Beziehungen zwischen den europaischen Grunfroschen und den Bastardcharakter von *Rana esculenta* L. (Anura).- Zool. Anz. 190 250-285.

GUNTHER, R. 1975a. Untersuchungen der Meiose bei Mannchen von *Rana ridibunda* Pall., *Rana lessonae* Cam. und der Bastardform *"Rana esculenta"* L. (Anura).- Biol. Zentralbl. 94 277-294.

GUNTHER, R. 1975b. Zum naturlichen Vorkommen und zur Morphologie triploider Teichfrosche, *"Rana esculenta"* L., in der DDR (Anura, Ranidae).- Mitt. Zool Mus. Berlin 51 145-158

GUNTHER, R. & S LUBCKE, 1979. Mitt. Zool. Mus. Berlin In druk.

HOTZ, H., 1974. Ein Problem aus vielen Fragen - europaische Grünfrosche (*Rana esculenta*-Komplex) und ihre Verbreitung.- Natur und Museum 104 262-272

MOORE, W.S., 1975. Stability of small unisexual-bisexual populations of *Poeciliopsis* (Pisces Poeciliidae).- Ecology 56 791-808.

SCHULTZ, R.J., 1969 Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates.- Amer. Natur. 103 605-619.

TUNNER, H.G., 1973. Das Albumin und andere Bluteiweisse bei *Rana ridibunda* Pallas, *Rana lessonae* Camerano, *Rana esculenta* Linné und deren hybriden.- Z. zool. Syst. Evolut. forsch. 11 219-233.

TUNNER, H.G , 1974. Die klonale Struktur einer Wasserfroschpopulation.- Z. zool. Syst. Evolut. forsch. 12 309-314.

UZZELL, T , 1963. Natural triploidy in salamanders related to *Ambystoma jeffersonianum*.- Science 139 113-115.

UZZELL, T. & L. BERGER, 1975. Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae* and their hybridogenetic associate, *Rana esculenta*.- Proc. Acad. Nat. Sci. Philadelphia 127 13-24.

UZZELL, T. & I.S. DAREVSKY, 1975. Biochemical evidence for the hybrid origin of the parthogenetic species of the *Lacerta saxicola* complex (Sauria Lacertidae), with a discussion of some ecological and evolutionary implications.- Copeia 1975 204-222.

UZZELL, T , L. BERGER & R. GUNTHER, 1975. Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia Salientia).- Proc. Acad. Nat. Sci. Philadelphia 127 81-91.

UZZELL, T., R. GUNTHER & L. BERGER, 1979. Mitt. Zool. Mus. Berlin. In druk.

VRIJENHOEK, R.C., R.A. ANGUS & R.J. SCHULTZ, 1978. Variation and clonal structure in a unisexual fish.- Amer. Natur. 112: 41-55.

WILBUR, H.M., 1971. The ecological relationship of the salamander *Ambystoma laterale* to its all-female gynogenetic associate.- Evolution 25: 168-179.

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STELLINGEN

1

Groene kikkers (*Rana esculenta* complex) zijn geen geschikte proefdieren.

Dit proefschrift.

2

De bewering van BLANKENHORN (1973), dat de drie vormen van het *Rana esculenta* complex goed te onderscheiden zijn op grond van verschil in vocalisatie, is ongegrond en hoogstwaarschijnlijk onjuist.

BLANKENHORN, H.J., (1973). Zum Stand der Forschung über die Verbreitung der Grünfrösche im Kanton Zürich.- Rev. Suisse Zool. 80, 656-661.

3

HAGSTRÖM's (1977) interpretatie van de botopbouw bij *Triturus* is onjuist.

HAGSTRÖM, T., (1977). Growth studies and ageing methods for adult *Triturus vulgaris* L. and *T. cristatus* Laurenti (Urodela, Salamandridae).- Zoologica Scripta 6, 61-68.

SMIRINA, E. & Z. ROČEK (1976). On the possibility ot using annual bone layers of alpine newts, *Triturus alpestris* (Amphibia, Urodela), for their age determination.- Věstn. Česk, spol. zool. 40, 232-237.

4

TRINKAUS (1973) houdt bij het bepalen van de snelheid, waarmee de diepe blastomeren zich tijdens de epibolie bij *Fundulus heteroclitus* voortbewegen, geen rekening met de snelheid van het substraat van deze cellen.

TRINKAUS, J.P., (1973). Surface activity and locomotion of *Fundulus* deep cells during blastula and gastrula stages.- Develop. Biol. 30, 68-103.

5

Het oecologisch onderzoek aan reptielen in Europa is duidelijk achtergebleven ten opzichte van dat in de rest van de wereld.

GANS, C. (1977). Biology of the Reptilia, Vol. 7, Ecology and Behaviour A. Academic Press, London. XVI + 720 pp.

6

De vooruitgang in het onderzoek van het crowding-effect bij Anura-larven wordt ernstig belemmerd door het gebrek aan standaardisering van de gebruikte methoden.

AKIN, G.C., (1966). Self-inhibition of growth in *Rana pipiens* tadpoles.- Physiol. Zool. 39, 341-356.
GROMKO, M.H., F.S. MASON & S.J. SMITH-GILL (1973). Analysis of the crowding-effect in *Rana pipiens* tadpoles.- J. Exp. Zool. 186, 63-72.
RICHARDS, C.M., (1962). The control of tadpole-growth by alga-like cells.- Physiol. Zool. 35, 285-296.
ROSE, S.M., (1960). A feedback mechanism of growth control in tadpoles.- Ecology 41, 188-199.

7

De conclusie van CHANG & HARTMANN (1972), dat door neerslag met sulfiden membraangebonden kwikverbindingen electronenmicroscopisch zichtbaar gemaakt worden, is vermoedelijk onjuist.

CHANG, L.W., & H.A. HARTMANN (1972). Electron microscopic histochemical study on the localization and distribution of mercury in the nervous system after mercury intoxication.- Exp. Neurol. 35, 122-137.

8

Bij het onderzoek naar DNAsen die betrokken zijn bij recombinatieprocessen in eukaryoten moet meer gebruik worden gemaakt van specifieke enzymtesten.

BURTON, W.G., R.J. ROBERTS, P.A. MYERS & R. SAGER (1977). A site-specific single-strand endonuclease from the eukaryote *Chlamydomonas*. - Proc. Natl. Acad. Sci. USA 74, 2687-2691.

9

Een proefschrift dient bij voorkeur te bestaan uit een bundel tijdschriftartikelen.

REID, W.M., (1978). Will the future generations of biologists write a dissertation? - Bioscience 28, 651-654.

Nijmegen, 1 februari 1979 H.E.J. Wijnands